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Toxicity and Anti-Tumor Effects of Cytarabine and Doxorubicin on Acute Myeloid Leukemia

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TOXICITY AND ANTI-TUMOR EFFECTS OF CYTARABINE AND DOXORUBICIN ON ACUTE MYELOID LEUKEMIA

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_____ _____

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ABSTRACT

Leukemia develops from the abnormal expansion of leukemic stem cells that have several genetic alterations that prevent these cells from differentiating into normal white blood cells. Acute myeloid leukemia (AML), one of the most aggressive types of leukemia, is characterized by the accumulation of large numbers of abnormal myeloid cells that do not differentiate into functional granulocytes or monocytes during the hematopoiesis process. The goal of this project was to study the anti-tumor effect of chemotherapy drugs Doxorubicin (Doxo) and Cytarabine (Ara-C) as inhibitors of leukemic cells expressing the oncogene *CBFB-MYH11*, which causes AML. This was accomplished by testing these drugs for specificity *in vitro* against rapidly dividing 3T3 cells and slowly dividing ME-1 leukemic cells expressing inv(16). The drugs were also tested *in vivo* establishing their toxicity in WT mice, and determining their efficacy in a leukemia transplantation mouse model. *In vitro* it was determined that Doxo and Ara-C affect the cell cycle of rapidly proliferating 3T3 cells, but have a lesser impact on slower dividing ME-1 cells. *In vivo* WT mice can tolerate up to 10 doses of 6 mg/kg/every other day of Doxo, and 27 doses of 200 mg/kg/day of Ara-C. Leukemic mice treated with chemo drugs 4 weeks after transplantation survived thirty nine days with treatment. However, mice treated only 1 week after transplantation survived only nineteen days with treatment. Our research shows that two important factors in decreasing the toxicity and increasing the efficacy of AML treatment are the amount of recovery time post irradiation and the concentration of drug dose. These therapeutic techniques may be used to expand our treatment options for AML.

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1.0 BACKGROUND

Leukemia is a malignant disease that affects the bone marrow and blood. Currently there is an estimated 231,461 people in the United States living with leukemia (Leukemia Facts and Statistics, 2009). The most common type of adult leukemia is acute myeloid leukemia (AML). Approximately 13,290 Americans were diagnosed with AML in 2008, and 1 out of every 5 children with leukemia were diagnosed with AML (Leukemia Facts and Statistics, 2009). Despite these intimidating statistics, successful treatment options are available for patients diagnosed with AML. One of these options is chemotherapy, which aims to kill as many dividing cells as possible and, over time, return blood cell counts to normal levels. This MQP report will focus on the toxicity and anti-tumor effects of two first line chemotherapy drugs, Cytarabine and Doxorubicin, in AML mouse models and cell lines.

1.1 Hematopoiesis

Blood cell formation arises from bone marrow stem cells, a process known as hematopoiesis. The bone marrow contains immature cells called hematopoietic stem cells (HSC) that will eventually mature into the cellular components of blood. The two main characteristics of HSCs are their self-renewal capabilities and their ability to differentiate into a variety of specialized cells. There are two different types of HSCs: one type is a long-term stem cell, which is self renewable over a long period of time. The second type is referred to as short-term stem/progenitor or precursor cell. These cells are immature cells, with reduced self renewal capacity, that are precursors to a fully differentiated cell of the same tissue type (Hematopoietic Stem Cells, 2009). While these progenitor cells are capable of proliferating, they do have a

limited ability to differentiate into more than one cell type. In normal blood cell development (**Figure 1**), HSCs develop and differentiate into myeloid or lymphoid progenitor cells, which undergo differentiation into red blood cells, platelets, and white blood cells. The pluripotent progenitor cell is the precursor for the multi-potential progenitors, the myeloid progenitor cells and the lymphoid progenitor cells. The myeloid progenitors (diagram left side) are precursor cells for red blood cells, platelets, granulocytes and monocytes. The lymphoid progenitors are precursors for B-lymphocytes, T-lymphocytes, and natural killer cells.

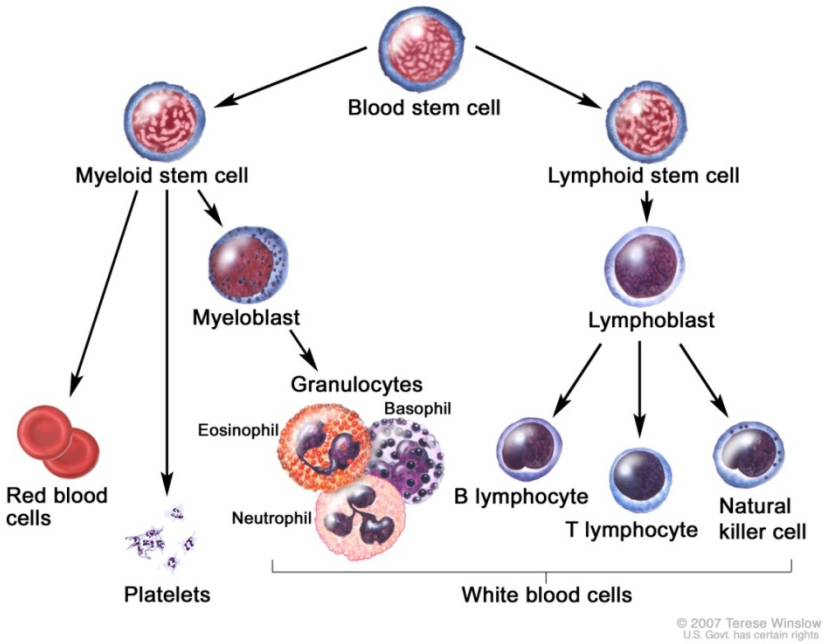


Figure 1: Diagram of Normal Hematopoiesis. This figure denotes the differentiation of hematopoietic stem cells into the cellular components of blood, an important process in leukemia (Chronic Lymphocytic Leukemia Treatment, 2008)

The process of hematopoiesis can be disrupted, and this can have a devastating impact on the human system. If HSCs do not differentiate into other types of normal stem cells, this can lead to leukemia, which is described in the following section.

1.2 Leukemia

Leukemia is cancer of the blood characterized by the production of irregular white blood cells in the bone marrow. Leukemia develops from the abnormal expansion of leukemic stem cells that have several genetic alterations that prevent these cells from differentiating into normal white blood cells. These immature leukemic stem cells also have rapidly proliferating cell cycles and usually a resistance to apoptosis.

1.2.1 Acute Myeloid Leukemia

While there are many types of leukemia, categorized by the level of maturation of white blood cells and the aggressiveness of the disease, acute myeloid leukemia (AML) is one of the most common types of adult leukemia. It begins in the bone marrow, but in the majority of patients with AML, this cancer spreads quickly to the blood and invades other parts of the body, specifically the lymph nodes, liver, spleen, central nervous system, and testes. AML is characterized by the accumulation of large numbers of abnormal myeloid cells that do not differentiate into functional granulocytes or monocytes during the hematopoiesis process (Bonnet and Dick, 1997). Granulocytes are white blood cells that contain enzymes that destroy microbes. There are three different types of granulocytes: neutrophils, basophils, and eosinophils, distinguished by the size and color of their granules. Monocytes are also white blood cells and form in the bone marrow to become blood-forming monoblasts, which eventually develop into mature macrophages (What is Acute Myeloid Leukemia?, 2007). Macrophages destroy bacteria and can recognize germs to help B-cells create antibodies to fight these cells. The types of blood cells mentioned above are shown below in **Figure 2**.

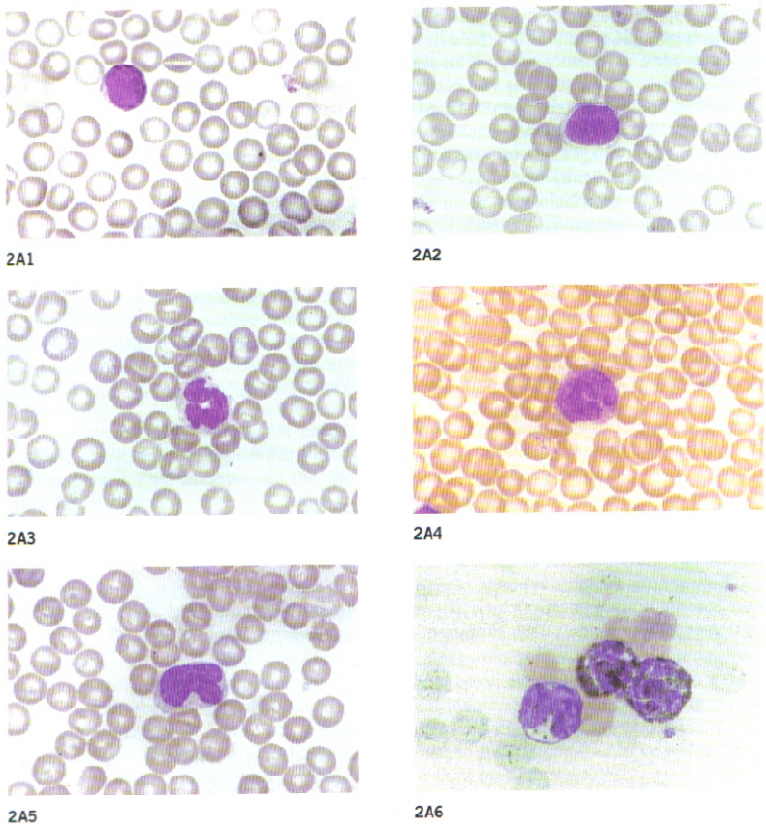


Figure 2: Photomicrographs of the Various Types of Normal White Blood Cells. Figure 2A1 is a small lymphocyte, with a platelet in the upper right hand corner. Figure 2A2 shows a large lymphocyte. Figure 2A3 is a neutrophil, figure 2A4 is an eosinophil, figure 2A5 is a monocyte, and figure 2A6 contains two neutrophils and a monocyte, on the left. (Fredrickson and Harris, 2000).

1.2.2 CBFB-MYH11 *Gene*

In the hematopoiesis process of an AML patient, the fusion oncoprotein CBFβ-SMMHC (smooth muscle myosin heavy chain) causes the development of pre-leukemia stem cells (PSC) from HSCs, which leads to the emergence of abnormal myeloid progenitors (AMP) that do not exhibit normal myeloid differentiation (Kuo et al, 2006). With additional mutations, AMPs become leukemia stem cells (LSC) that expand into AML. This process is shown in **Figure 3**.

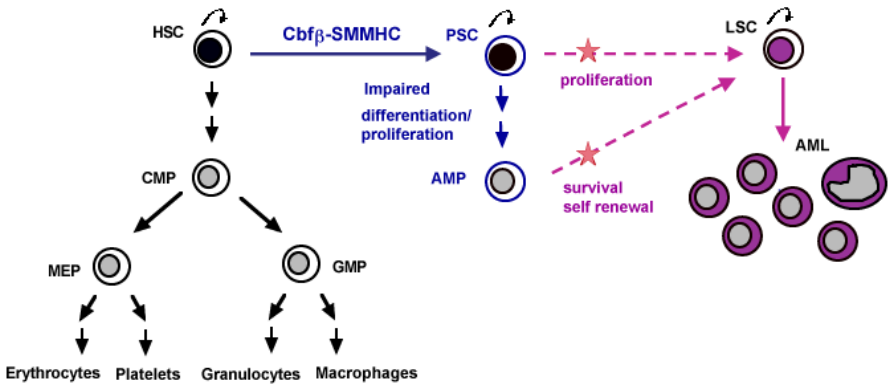


Figure 3: Development of Acute Myeloid Leukemia. Diagram shows the development of AML from hematopoietic stem cells (Castilla, n.d)

Clonal chromosomal abnormalities in blast cells from leukemia patients have shown that AML can develop from chromosomal translocations that mainly affect transcription factors. The heterodimeric transcription complex core-binding factor (CBF) is a regulator of gene expression during blood cell development. It consists of a DNA binding α subunit, encoded by one of the RUNX genes, and a β subunit, encoded by the *CBFB* gene (Castilla, 2006). The CBF factor is the most common target of chromosomal rearrangements in AML, specifically the inversion of chromosome 16 [inv(16)] (Castilla, 2006). Approximately 12% of AML patients carry the inversion in chromosome 16 (Castilla, 2006). *CBFB* forms a heterodimer with RUNX1 (runt-related transcription factor 1), which is a heterodimeric transcription factor that binds to the core element of several promoters and enhancers (Runt-Related Transcription Factor 1, 2009). Translocations, such as the inv(16) mutation, alter the RUNX1 and *CBFB* genes to create fusion proteins that have dominant effects in the development of leukemia. The fusion gene *CBFB-MYH11*, which encodes the CBFβ- SMMHC fusion protein, is present in almost all cases of subtype M4 AML (Castilla, 2004).

1.2.3 Treatment for AML

There are several different treatment options for patients suffering from AML. The best treatment option depends on the subtype of AML and the cytogenetic study of the leukemic cells. However, the major treatment used for patients with AML is chemotherapy. Chemotherapy treatments are generally divided into two phases: remission induction and post-remission therapy (consolidation) (What is Acute Myeloid Leukema?, 2007). The purpose of remission induction is to destroy all leukemic cells. This is accomplished by treatment with a combination of two chemotherapy drugs, Ara-C and an anthracycline drug, such as daunorubicin. Since remission induction does not often kill all leukemic cells, a second treatment phase, consolidation, is needed to fully treat the leukemia and prevent relapses. One of the consolidation treatments for AML usually includes several cycles of high dose Ara-C therapy (What is Acute Myeloid Leukemia?, 2007). This therapeutic option has resulted in remission four years post-treatment in 40% of patients with AML under 60 years of age (What is Acute Myeloid Leukemia?, 2007). However, *CBFB-MYH11*-positive AML cases are most frequently seen in patients over 60 years of age. The overall five year survival of AML patients over 60 years of age with *CBFB-MYH11* is 20%. Therefore, intensive therapies are not recommended for this age group.

1.2.4 Mouse Models

The purpose of mouse model systems in leukemic research is to provide researchers with a platform to develop and explore therapeutic approaches for treating different types of leukemia. These models provide a controlled environment where therapies that would be impossible or difficult to be tested on humans can be analyzed. Mice are useful because they can be designed to have certain phenotypic or genotypic characteristics that can serve as a control. The laboratory

mouse, *Mus musculus*, is one of the best model systems for cancer investigations due to various factors that include: its small size and propensity to breed in captivity, lifespan of 3 years, extensive physiological and molecular similarities to humans, and an entirely sequenced genome (Frese and Tuveson, 2007).

With respect to leukemia, there are many specific advantages to using mice as hematopoietic cancer models. One advantage is the genetic diversity of the mice can be regulated to mimic some of the genetic mutations known to induce the disease. Some types of acute leukemia involve several cooperating mutations; however to facilitate an investigation of the role of each mutation, mouse models can be designed to only have one mutation within its germline. Alternatively, multiple oncogenic mutations can be designed into the model by crossbreeding or other strategies (Braun et al., 2008). Another advantage to using mice as hematopoietic cancer models is there are widespread similarities between human and murine hematopoiesis, so the effects of oncogenic mutations on the mouse model may be similar to the effects shown in humans. In addition, hematopoietic malignancies are almost always transplantable into mouse models (Braun et al., 2008). As a result of these reproduced malignancies, controlled experiments can be performed *in vivo* to understand the effects of therapeutic techniques on the mouse's system. A final advantage is the clear disease endpoints present in a mouse model. Intermediate endpoints of clinical appearance, peripheral blood counts, and/or lymphadenopathy are all clear indicators of the effects of therapeutics on the progression of the disease (Braun et al., 2008). Overall, using mice as hematopoietic cancer models has proven to be very useful in discovering new methodologies for the detection, management and treatment of cancer in humans.

This MQP project will utilize a mouse model that was transplanted with leukemic cells that carry the inv(16) mutation, as this mutation expresses the fusion gene *CBFB-MYH11*. This lab uses conditional *CBFB-MYH11* knock-in mouse models to show that this gene produces AMPs that will lead to AML, along with other mutations that can provide proliferation and/or survival advantage of the AML cells (Castilla, n.d.). The leukemic cells transplanted into the 129 SvEv mouse models in this project were collected from the peripheral blood, bone marrow, and spleen of the *CBFB-MYH11* knock-in mouse model, to ensure that the inv(16) mutation was present and AML was expressed. The mice transplanted with leukemic cells provide a solid model to study the effect of treatment with chemotherapy drugs on AML.

1.3 Chemotherapy Drugs

Chemotherapeutic drugs were first introduced in 1945, when research was done on nitrogen mustards and its use in the treatment of leukemias and other cancers. Since that time, over sixty drugs have been registered in the United States as treatments for cancer, and although significant strides have been made, there are still many problems with these drugs that need further improvement, such as high toxicity and low specificity. One of the main problems is that many anticancer agents evoke different responses in different types of cancers. Once a new agent has been identified, and testing shows that it is effective in treating one type of cancer; great effort is devoted to test the same drug in various combinations for different classes of cancer. While this course of action has led to some advances in the treatment of many cancers, significant increases in survival will only occur if selectivity of present-day anticancer agents can be increased or new classes of more selective agents can be discovered. This is a difficult task because currently, no existing laboratory tests can accurately predict which chemical will be

effective against a particular class of human cancer. Tests can only confirm or deny a compound's anticancer property. Due to these shortcomings in research and the development of new drugs that specifically target certain classes of cancers, as a general trend, cancer mortality has changed little over the past forty years (Connors, 1996).

Another problem which arose in the field of chemotherapy was the toxicity of most anticancer drugs. One of the promising fields of research by medicinal chemists is the development of analogs of anthraquinones with lower toxicity that maintain the anticancer effect. Although considerable research efforts have decreased the cytotoxic effects of many cancer drugs, toxicity remains a problem that hinders cancer therapy.

1.3.1 Doxorubicin

Doxorubicin (Doxo) is an anthraquinone anticancer agent composed of an amino sugar (daunosamine) linked by an *O*-glycosidic bond to an aglycone (doxorubicinone) (**Figure 4**). The drug has been used for more than 20 years in the treatment of patients with certain types of leukemias, lymphomas, soft tissue sarcomas, as well as cancers of the bladder, breast, stomach, lung, ovaries, thyroid, multiple myeloma, and others (Lipshultz, 1991).

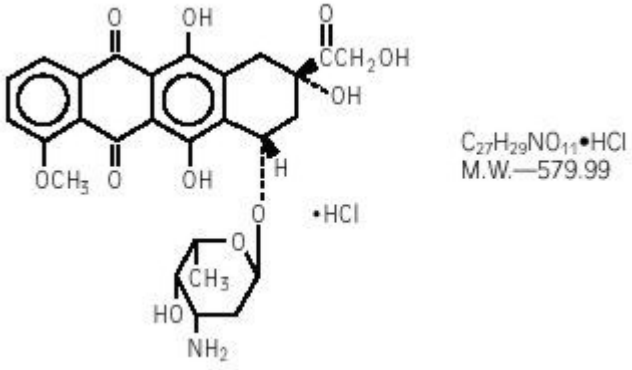


Figure 4: Diagram of the Chemical Structure of Doxo. (Doxorubicin, n.d.)

The exact mechanism of action of Doxo is complex and still somewhat unclear, though it is thought to interact with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of the enzyme topoisomerase II, which unwinds DNA for transcription (Gerwitz, 1999). Doxo stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication (Ping-Hu, 1996). The planar aromatic chromophore portion of the molecule intercalates between two base pairs of the DNA, while the six-membered daunosamine sugar sits in the minor groove and interacts with flanking base pairs immediately adjacent to the intercalation site, as evidenced by several crystal structures. (Ping-Hu, 1996).

1.3.2 Treatment With Doxo

Doxo is rapidly and broadly distributed in humans as a chemotherapy treatment, and accumulates in irrigated tissues such as liver, lung, kidney, etc. and especially heart. During the past three decades, a standard of care regimen with doxorubicin has been established for AML patients. Typically, patients have been treated with the cell-specific agent Ara-C 100 mg/m^2/d by continuous infusion for 7 days, and Doxo at a dosage of 45 to 60 mg/m^2/d intravenously for 3 days (Tallman, 2005). A plethora of studies have tested various dosages, combination treatments, and/or standard therapy followed by high doses of Ara-C. But even with all the testing and research, "the optimal dose, schedule and number of cycles of consolidation chemotherapy for patients with AML who achieve CR have not been established" (Tallman, 2005). Despite reported advantages of any one laboratory tested approach, no approach has proven to be definitively better than the standard drug combination regimen described previously, which is described in the table below.

Table 1. Therapeutic Strategies in AML and Relapsed or Refractory AML

Strategy	Comments
AML	
Induction	
Daunorubicin 45-60 mg/m^2 for 3 days or alternative anthracycline or the anthracenedione mitoxantrone with cytarabine 100 mg/m^2 for 7 days	Optimal dose of anthracycline is unknown.
	No definitive evidence that any anthracycline or the anthracenedione mitoxantrone is better at any age.
	Standard regimen is effective in all cytogenetic subtypes.
	No evidence that addition of high-dose cytarabine (HiDAC) or etoposide is essential.
	The induction regimen should not be attenuated for older adults.
After remission*	
HiDAC 1-3 g/m^2 over 1-3 hours for 3-6 days x 1-4 cycles	Optimal dose, schedule, and number of cycles of HiDAC are unknown.
	Although HiDAC is clearly effective, groups using different intensive regimens have reported similar data.
Maintenance therapy	Standard of care in acute promyelocytic leukemia (APL); role in other subtypes is less convincing.
Stem-cell transplantation	Most potent antileukemic strategy, but caution is warranted in interpretation of studies that are underpowered and often not applicable to current practice.
Relapsed or refractory AML	
Chemotherapy	Rarely curative in any subtype.
Effective reduction in leukemia-cell burden	Essential for cure.
	High-dose cytarabine is most effective.
	No evidence that additional drugs are beneficial.
	May be effective even if prior exposure to cytarabine in induction or consolidation.

	Best if first CR is long (more than 6-12 months).
	Investigational approach is appropriate if short CR1 or refractory.
Allogeneic transplantation	Potentially curative.
	Best results if in second CR or in early first relapse.
Autologous transplantation	Few reports of cure.
	Best results if in second CR with previously harvested stem cells.
Palliative care	Appropriate for older adults not eligible for curative approaches.

(Tallman, 2005)

Currently, with this standard of treatment, approximately 50% to 70% of AML patients achieve complete remission (CR). Unfortunately, long term disease-free survival (DFS) is only experienced for 20% to 30% of patients, and the majority of patients die from persistent or relapsed AML. In an analysis of 3000 AML patients enrolled in 5 successive clinical trials with Ara-C and Doxo for induction and intensive post remission therapy, 62% of patients achieved CR, however 76% relapsed or died from the disease. Fortunately, overall survival rates have improved since the 1970s. The five year overall survival rate of 2000 patients under the age of 55 has increased from 11 % in the 1970s to 37% in the 1990s (**Figure 5**).

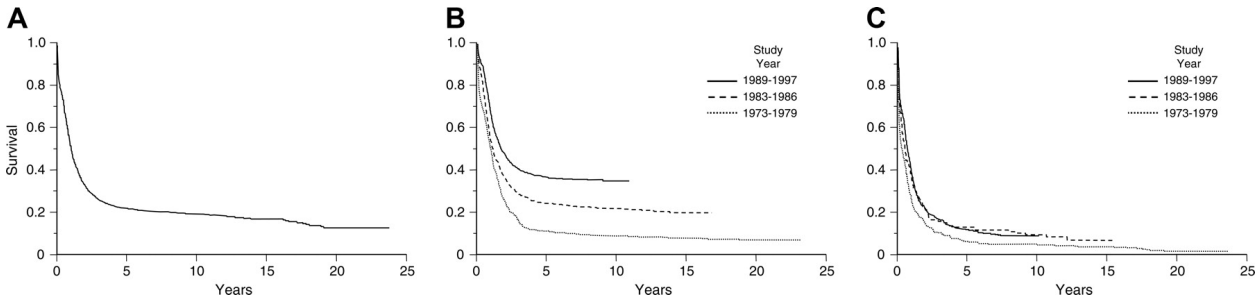


Figure 5: Estimates of Overall AML Survival. (A) Kaplan-Meier product-limit estimate of overall survival for patients with newly diagnosed AML treated on ECOG protocols between 1973 and 1997. (B) Kaplan-Meier product-limit estimate of overall survival for younger patients (ages ≤55 years) with newly diagnosed AMLtreated on ECOG protocols between 1973 and 1997. (C) Kaplan-Meier product-limit estimate of overall survival for older patients (ages > 55 years) with newly diagnosed AML treated on ECOG protocols between 1973 and 1997.

In 1973, Charlotte Tan, a renowned oncologist at the Sloan-Kettering Cancer Center in New York, published a ground breaking paper on the antitumor effects of Doxo. Adriamycin, another commercial name of Doxo, was given to 234 patients with leukemia and other types of neoplastic diseases. The dose in children was 0.5 mg/kg daily to a total of 2.4 mg/kg per course. In adults, the single dose was 0.4 mg/kg to a total of about 2.5 mg/kg in 10 days. In children with acute leukemia previously treated, adriamycin produced complete remissions (12%) and good partial remissions (26%). Tumor regressions were seen in 60% of the patients with solid tumors. These included lymphoma, embryonal rhabdomyosarcoma, neuroblastoma, Ewing's sarcoma, Wilms' tumor, ovarian tumor, hepatoma, embryonal carcinoma, malignant teratoma, and retinoblastoma. The therapeutic responses in adults were less consistent, and confined to lymphomas and soft tissue sarcomas. Varying degrees of transient electrocardiographic changes had been seen in children treated. Adriamycin may have contributed to cardiac failure and death in one child, who had recurrent pulmonary metastases.

1.3.3 Doxo Side Effects

While Doxo is used as a primary chemotherapy agent, it also has many adverse side effects. Acute side-effects of Doxo can include nausea, vomiting, and heart irregularities. It can also cause neutropenia, a decrease in white blood cell count, as well as complete alopecia (hair loss). When the cumulative dose of Doxo reaches 550 mg/m², the risks of developing cardiac side effects, including heart failure, dilated cardiomyopathy, and death, dramatically increase. Doxo cardiotoxicity is characterized by a dose-dependent decline in mitochondrial oxidative phosphorylation. Reactive oxygen species, generated by the interaction of Doxo with iron, can then damage the cardiomyocytes (heart cells), causing myofibrillar loss and cytoplasmic vacuolization. Accordingly, from the pharmacokinetic point of view, its cardiotoxicity can be explained in terms of massive binding in the myocardium, justifying interest in studying the distribution of Doxo in different organs and tissues, and especially in the heart (Singal, 1998). Unfortunately, the clinical use of Doxo is limited by its toxic effects, such as cumulative dose-related cardiotoxicity, myelosuppression, and the development of drug resistance (Singal, 1998). Additionally, some patients may develop "Hand-Foot Syndrome," characterized by skin eruptions on the palms of the hand or soles of the feet that are accompanied by swelling, pain and erythemia. Due to these side effects and its red color, Doxo has earned the nickname "red devil" or "red death".

1.3.4 Doxorubicin Treatment in Mouse Models

As previously described, the most effective dose, cycle, and combination of chemotherapy has not been established for patients with AML. In order to accomplish this goal, research must first be done in the mouse model before proceeding to clinical trials. In a 2005

publication by Brian Lee, mice transplanted with leukemia were tested with various doses of Doxo in order to "increase survival" (Lee et al, 2006). It was found that in the AML "mouse model, the highest tolerated dose of Doxo is 3 mg/kg for three days" (Lee et al, 2006). Further studies will be necessary to establish the most effective treatment for AML in the mouse model, and eventually in AML patients.

1.3.5 Cytarabine

Cytarabine, commonly known as Ara-C, is a chemotherapy agent used primarily for the treatment of hematological cancers, such as non-Hodgkin lymphoma and acute myeloid leukemia. This agent was first discovered in the 1960s in Europe, and it was not approved for use in the US until nine years later, in 1969. Figure-6 shows the chemical structure of Ara-C.

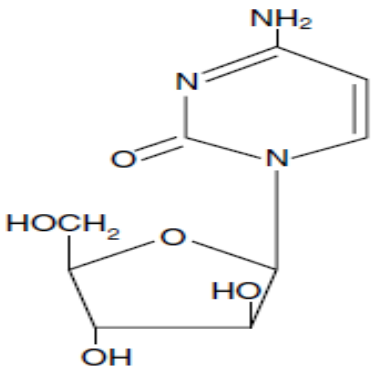


Figure 6: Diagram of the Chemical Structure of Ara-C. (DepoCyt, n.d.)

Ara-C is used in standard induction regimens and consolidations/maintenance therapy after remission (Yin, 2005). The drug "is phosphorylated to nucleotide" form, 1-β-D-arabinofuranosyluracil (Ara-U) by the pymidine nucleoside deaminse (cytidine aminohydrolase), which damages DNA during the S phase of the cell cycle (Dedrick et al, 1973). It also inhibits both DNA and RNA polymerases and nucleotide reductase enzymes which are needed for DNA

synthesis. Any rapidly dividing cells, cancerous or normal, which need DNA replication for mitosis, are the most affected by the drug (Yin, 2005) . Studies with Ara-C have shown that it is "active only against dividing cells, and does not cause the death of nondividing cell *in vitro*, even at extremely high concentrations (up to 2500 ug/ul)" (Leach, 1969).

Ara-C is administered in the body through continuous intravenous (i.v.) infusion. The drug is metabolized in the liver, kidney, GI mucosa, and granulocytes. The drug, when administered intravenously, has an initial distribution half-life of about ten minutes. During the initial half life, the majority of the drug is metabolized in the kidney, liver and gastrointestinal tract into its inactive metabolite, uracil arabinoside (Dedrick et al*.,* 1973*)*. The secondary elimination half life is longer, and lasts one to three hours. The majority of the dose administered is excreted via the kidney within one day.

This chemo agent is also very toxic to the human body, as well as to mouse models. Some toxic effects of the drug are: leucopenia, thrombocytopenia, anemia, GI tract abnormalities, fever, conjunctivitis, and pneumonitis (Yin, 2006).

Due to its importance as a chemotherapy agent, further research must be conducted to establish the most effective dose and schedule of Ara-C in the AML mouse model. It is important to establish the highest tolerated dose, with minimum toxicity and maximum efficacy at killing AML cells. From this research, a better therapy and schedule can be developed for patients suffering from AML.

1.3.6 Treatment With Ara-C

In AML patients, there are two different courses of chemotherapy: induction remission and consolidation. In induction remission, the patient is treated with one or two sessions of Ara-

C followed by treatment with daunorubicin. In post remission therapy, often called consolidation, the patient is only treated with Ara-C.

1.3.7 Ara-C Treatment in Mouse Models

As previously described, the most effective dose, cycle, and combination of chemotherapy has not been established for patients with AML. In order to accomplish this goal, research must first be done in the mouse model before proceeding to clinical trials. In previous studies done with Ara-C, the mouse can be tolerate a treatment of 100 mg/kg every day for 9 days, followed by a 7 day rest period, and continued with a second session of 9 injections (Largaespada, personal communication). Further studies need to supplement this finding to establish the most effective treatment for AML in the mouse model, and eventually in AML patients.

2.0 PROJECT PURPOSE

As described in the Background section, although Doxo and Ara-C chemotherapeutic agents have been shown to be somewhat effective at treating AML in mouse models and patients, both drugs still show considerable toxic side effects, and patient survival is limited. Thus, the most effective dose, cycle, and drug combination have not yet been established. The goal of this project was to further study the anti-tumor effect of Doxo and Ara-C *in vivo* in an AML mouse model expressing oncogene *CBFB-MYH11* and in WT mice, and *in vitro* against the rapidly dividing 3T3 cell line and against the slowly dividing, human leukemic ME-1 cell line expressing inv(16).

3.0 METHODS

3.1 *In Vitro* Drug Testing in 3T3 and ME-1 Cell Lines

3.1.1 Propidium Iodide Analysis

The propidium iodide (PI) flow cytometric assay has been widely used for the evaluation of phases of the cell cycle in different experimental models. It is commonly used to determine the percentage of cells in each phase based on the principle that these cells are characterized by DNA fragmentation (Riccardi and Nicoletti, 2006). Use of a fluorochrome, such as PI, that is capable of binding and labeling DNA makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis, and subsequent identification of hypodiploid cells (Riccardi and Nicoletti, 2006).

The PI stain can distinguish cells at certain points along the cell cycle; three histogram markers identify each phase of the cell cycle, which include the G0/G1 phase, S phase, and G2/M phase, with an optional fourth marker that can identify cells in an apoptotic state (Cell Cycle Assay, 2009). **Figure 7** shows the state of growth and division of cells at each phase of the cell cycle. The apoptotic phase is not shown in the figure; however it is characterized by less than 2N DNA. Absolute counts are established in this essay to determine whether changes in the cell cycle result from drug treatments, or are caused by changes in cell number due to cell proliferation or cell death (Cell Cycle Assay, 2009). In this project, the purpose of using the PI assay was to determine the effects of Doxo and Ara-C on the cell cycle.

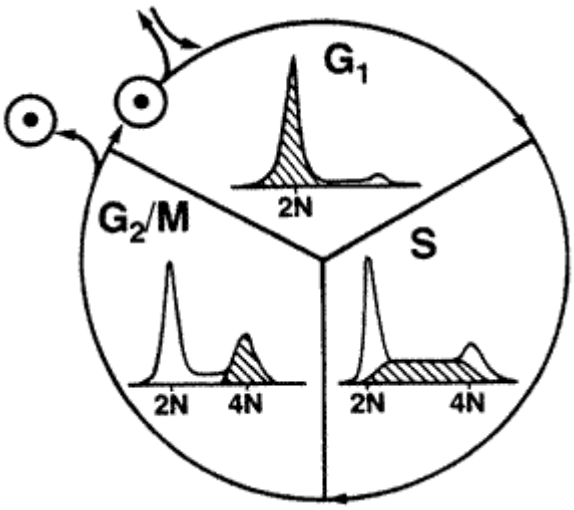


Figure-7: The Phases of the Cell Cycle. (The Phases of the Cell Cycle, 2008).

3.1.2 Cell Lines

The two cell lines used in this assay were the mouse NIH-3T3 cells and the human ME-1 cell line. The 3T3 cell line is a mouse fibroblast cell culture established from disaggregated tissue of an embryonic albino Swiss mouse (*Mus musculus*). When these cells were developed in the 1960s by George Todaro and Howard Green, they were used to show the difference between cell mortality and a cell's ability to undergo oncogenic transformation (Hill, 2007). This is a rapidly growing cell line, and should be strongly affected by Doxo or Ara-C treatments.

The ME-1 cell line was established from peripheral blood leukemia cells of a patient with acute myelomonocytic leukemia with eosinophilia (M4E0) (Yanagisawa et al, 1991). This cell line carries the chromosome abnormality inv(16) (p13; q22) expressing *CBFB*-MYH11, among other mutations. ME-1 cells were found to be monoblastic when cultured in RPMI 1640 medium with 10% fetal bovine serum. However, with the addition of the cytokines, interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-4, the cells differentiate to

macrophage-like cells (Li, 2008). The proliferation and differentiation of ME-1 cells by IL-3 or GM-CSF are related to the activation of protein kinase C, whereas those by IL-4 involve other regulatory systems (Durst, 2003). ME-1 cells are useful for studying the pathogenesis of M4E0 and the mechanisms of proliferation and differentiation of leukemic and normal progenitors by cytokines.

3.1.3 3T3 Cell Culture

3T3 cells were plated in a 6-well plate at $2x10^5$ cells per well containing a total of 3 mL of cells and Dulbecco's Modified Eagle Medium (DMEM) media containing antibiotic. After a 24 hour incubation period at 37ºC, Doxo and Ara-C were added to each well. Doxo stock solution (2 mg/mL) was added to three wells at volumes of 100 µL, 10 µL, and 1 µL respectively. Ara-C was diluted 10X (to 10 mg/mL) before it was added to two wells at volumes of 100 µL and 10 µL. Nothing was added to the final well, which served as a negative control. After a second 24 hour incubation period with the chemotherapy drugs, the cells were prepped for PI analysis.

3.1.4 ME-1 Cell Culture

ME-1 cells were plated in nine wells in a 96-well plate with 50,000 cells per well. After a 24 hour incubation period at 37ºC, the chemotherapy drugs were added to each well. The wells contained four different concentrations of Doxo and Ara-C, and a control for each drug only containing ME-1 cells. Ara-C was diluted 10X from a powder to a final concentration of 100 mg/mL. A serial dilution was made for Doxo at concentrations of 2 mg/mL, 0.4 mg/mL, 0.08 mg/mL and 0.016 mg/mL, and Ara-C at concentrations at 10 mg/mL, 2 mg/mL, 0.4 mg/mL and

0.08 mg/mL. Each well contained 200 µL of ME-1 cells followed by 6.7 µL of the diluted concentration of either Doxo or Ara-C. Both Doxo and Ara-C were resuspended in the wells to avoid the formation of precipitates. After the cells had been treated with Doxo and Ara-C for 24 hours, they were prepped for PI analysis.

3.1.5 Leukemic Cell Culture

Leukemic cells were obtained from mice that had AML. These cells were harvested from the spleen, bone marrow, and peripheral blood of these mice. The leukemic cells were plated in nine wells in a 96-well plate with 50,000 cells per well. After a 24 and 48 hour incubation period at 37ºC, the chemotherapy drugs were added to each well. The wells contained four different concentrations of Doxo and Ara-C, and a negative control for each drug only contained leukemic cells. Ara-C was diluted 10X from a powder to a final concentration of 0.1 grams/mL. A serial dilution was made for Doxo at concentrations of 2 mg/mL, 0.4 mg/mL, 0.08 mg/mL and 0.016 mg/mL, and Ara-C at concentrations at 10 mg/mL, 2 mg/mL, 0.4 mg/mL and 0.08 mg/mL. Each well contained 200 µL of leukemic cells followed by 6.7 µL of the diluted concentration of either Doxo or Ara-C. Both Doxo and Ara-C were resuspended in the wells to avoid the formation of precipitates. After the cells had been treated with Doxo or Ara-C for 24 and 48 hours, they were prepped for PI analysis.

3.1.6 Preparation of Cells for PI Analysis

The media and cells of each well was extracted and spun down at 1,700 g for 10 minutes. The supernatant was then aspirated. Next, 300 µL of phosphate buffered saline (PBS) was added to resuspend the cell pellet. The cells and PBS were then transferred drop by drop into 5 mL

tubes containing 2.7 mL of 70% ethanol in order to fix the cells in whatever phase of the cycle they were in. Samples were then sent to the FACS facility where PI was added and analysis was performed.

3.2 In Vivo Drug Testing-Toxicity of Doxo and Ara-C in WT Mice

Toxicity experiments were performed to determine the toxicity of Doxo and Ara-C on normal proliferating cells and the highest tolerated dose (HTD) of each drug. The toxicity experiments were tested on 129 SvEv mice. The 129 SvEv mouse is a subline of the 129/J strain. The 129/J mouse and its sublines have been used to derive most of the commonly used embryonic stem cell lines (Reiners and Singh, 1997). This is an agouti coat-colored murine strain. The toxicity experiments using Doxo in this project were based on previously published studies indicating this drug can be tolerated at an injection of 3 mg/kg every 3 days over a period of 7 days (3 total injections) (Lee et al, 2006). The toxicity experiments using Ara-C were based on previous studies indicating this drug can be tolerated at 100 mg/kg every day for 9 injections, followed by a 7 day rest period, and continued with a second session of 9 injections (Largaespada, personal communication).

The 100 mg/mL Ara-C stock was diluted 1:100 with PBS to a final concentration of 1 mg/mL. The Doxo was also diluted 1:100 with PBS from its original 2 mg/mL serum form, to a final concentration of 0.02 mg/mL. The 129 SvEv mice were injected intraperitoneally (i.p.) through the course of treatment until they died or it was no longer humane to continue with injections. At this point, the mice were euthanized using IACUC protocol A-1266 approved by UMASS Medical School.

3.2.1 Retro-Orbital Sinus Bleeding

To obtain blood for CBC, PI, and FACS analyses, retro-orbital sinus bleeds were performed. First, the mouse was anesthetized using 400-500 µL of Avertin. The mouse was then held with the forefinger and the thumb tightly at its neck to have access to the eye. To collect the blood, small capillary tubes were inserted into the venous sinus laterally at approximately a 45º angle. Once the tube entered the peri-orbital venous sinus that fills the bony orbit of the eye of the mouse, blood would begin to flow through capillary action (Mouse: Retro-orbital, 2006). Once the desired amount of blood was collected, the bleeding was stopped by applying pressure with gauze.

*3.3 In Vivo D*rug Testing: Efficacy and Toxicity of Doxo and Ara-C in the Leukemia Transplantation Model

Experiments were performed with the leukemia transplantation mouse model to determine the toxicity and anti-tumor effects of Doxo and Ara-C on AML cells *in vivo*. Leukemic cells were harvested from the spleen, peripheral blood, and bone marrow of a mouse that had AML caused by *CBFB-MYH11* expression. These leukemic cells were cryopreserved and then thawed when needed. In these experiments, $5x10^5$ leukemic cells were transplanted into each mouse. The mice used were first irradiated using 650 rads to kill normal bone marrow. The leukemic cells were then injected intravenously (i.v) into one of the lateral tail veins of the mouse. The mice were given at least one week to recover from the irradiation before beginning treatment. Treatment was administered using the same protocol as the toxicity experiments in WT mice.

3.3.1 FACS Analysis

Fluorescence activated cell scanning (FACS) was used to demonstrate the presence of malignant cells in peripheral blood and bone marrow suspensions. During these experiments FACS analysis was used to identify the progression of leukemia in mouse peripheral blood, spleen cells, and bone marrow. Peripheral blood was collected through retro-orbital bleeding into 2mL Eppendorf tubes containing 1mL of red blood cell (RBC) lysis buffer. The cells were left in the RBC lysis buffer for 20 minutes to ensure cell lysis occurred followed by five minutes of centrifugation at 1,200 g. The supernatant was aspirated and this process was repeated. FACS buffer was added to each tube depending on the size of the pellet and the number of samples needed. Enough FACS buffer was added so that each sample tube contained approximately 50 µL of solution.

In this FACS analysis, antibodies tagged with a fluorescent dye bind specifically to receptors on desired cells to determine if leukemia is present in the blood. The primary antibodies used in these experiments were labeled with phycoerythrin (PE), one of the most common fluorescent dyes used in FACS analysis. These antibodies specifically recognized the following cell surface markers: cKit, Gr1, B220, Mac1, and CD3. cKit is present in all leukemic cells, and this is an important cell surface marker used to identify certain types of hematopoietic (blood) progenitors in the bone marrow. Specifically hematopoietic stem cells (HSC), multipotent progenitors (MPP), and common myeloid progenitors (CMP) express high levels of cKit. For this experiment cKit was diluted 1:32 with FACS buffer, Gr1, B220, Mac1 were diluted 1:16 with FACS buffer, and CD3 was diluted 1:8 with FACS buffer. The blood samples were then put on ice for 20 minutes for staining to occur. The antibodies are light sensitive so it was necessary to cover them during this 20 minute period. After this incubation period 1 mL of

FACS buffer was added to the samples to wash away any antibodies that had not bound. Then the cells were once again centrifuged, and a pellet was obtained. Finally 500 µL of fixing buffer (10% formaldehyde in FACS buffer) was added to the pellet and mixed well. The samples were transferred into 5mL tubes and sent to the FACS facility for analysis.

FACS analysis using spleen and bone marrow cells followed the same procedures as the preparation for peripheral blood cells. Spleen cells and bone marrow were obtained after mice died or were euthanized. Spleen cells were harvested from the deceased mice, crushed using a pestle and mortar in RBC lysis buffer, filtered, then spun down to prepare the cells for FACS analysis. Bone marrow was harvested from the femur and tibia bones of the mouse. The bones were dissected from the muscles and tendons, and placed in a petri dish containing RBC lysis buffer. Similar to the spleen cells, the bone marrow was then crushed into a paste and passed through a filter, and centrifuged to prepare the cells for FACS analysis using the procedures outlined above.

3.3.2 Blood Smears

Blood smears were created to study the morphology of the blood cells of the mice. First, a small drop of blood was obtained with a capillary tube from the orbital sinus of the mouse. The blood droplet was placed on a slide and spread across the surface using a second slide. The slide was left to dry for a period of time before staining. Wright Giemsa Staining was used to prepare the slide. In order to stain the slide, Coplin jars were prepared with the necessary solutions. Jar 1 contained fresh methanol, jar 2 contained 30 mL of Wright Giemsa Stain, jar 3 contained 5 mL Wright Giemsa Stain, 0.4 mL of Azure Blend, and 30 mL of ddH_20, and jar 4 contained 3-5 mL of Wright Giemsa Stain and 30mL pH 6.8 phosphate buffer. The slides were first fixed in

methanol for 30 seconds. After each slide was placed in a particular jar, the slides were dabbed onto paper towels to soak up excess liquid. Next, the slides were placed in jar 2 for 3 minutes, followed by jar 3 for 10 minutes, and jar 4 for 2 minutes. Finally slides were placed into a jar with distilled water to remove excess stain, then both sides of the slides were washed gently under distilled water. The slides were air dried for several minutes before being viewed under a microscope.

4.0 RESULTS

Chemotherapy is the current method of treating AML. The purpose of the first round of chemotherapy treatments is remission induction to normalize blood counts and to eliminate as many leukemic cells as possible from the blood and bone marrow. Currently, the most common treatment regimen for AML is chemotherapy with Doxo and/or Ara-C. However, these drugs have proven to be extremely toxic as they not only target preferentially expanding cells in AML but also normal proliferating cells. The most effective dose, cycle, and drug combination in mice and cell lines has not yet been established. In order to further study the anti-tumor effect of Doxo and Ara-C as inhibitors of leukemic cells expressing the leukemia oncogene *CBFB-MYH11*, we tested the drugs for their *in vitro* effect on the growth, apoptosis, and differentiation of ME-1 inv(16) cells compared to 3T3 cells, and *in vivo* established the toxicity of Doxo and Ara-C in wild type mice to determine the maximum tolerated concentration (MTC), and established the efficacy of leukemia drugs in the leukemia transplantation model.

4.1 *In Vitro* Drug Testing: PI Staining and FACS Analysis

In order to determine the effects of Doxo and Ara-C on the cell cycle, Propidium Iodide (PI) staining was done on 3T3 cells and ME-1 cells, treated with various concentrations of the drugs and analyzed by FACS after 24 hours of incubation. The PI stain can distinguish cells at the G0/G1 phase, S phase, G2/M phase, and an apoptotic state (Cell Cycle Assay, 2009). 3T3 cells are rapidly proliferating cells, and ME-1 are slow-proliferating cells that contain the inv(16)mutation. It was initially expected that the chemo drugs would have a greater effect on the ME-1 cell line, a human leukemic cell line, as opposed to the 3T3 cell line.

Figure 8 shows the PI staining and FACS analysis of 3T3 cells treated for 24 hours with volumes of Doxo at 100 µl, 10 µl, and 1 µl at a concentration of 2 mg/mL, and volumes of Ara-C at 100 µl and 10 µl at a concentration of 10 mg/mL, and control cells. The percentage of cells in each phase of the cell cycle is shown in **Table II**. Doxo at high concentrations has a very cytotoxic effect on 3T3 cells, killing the cells before they enter the cell cycle. In panel A, the majority of cells are in an apoptotic state (left peak), and as the concentration of Doxo decreases (B, C), the cells are able to progress further through the cell cycle (middle and right peaks). In parts D and E, Ara-C has a very cytostatic effect on 3T3 cells by preventing the cells from proliferating, holding them in the G1 phase. Very few cells progressed to S or G2/M. The control (F) shows that although the majority of cells are in the G1 phase, a number of cells were able to progress along the cell cycle to S phase and G2/M (right peaks).

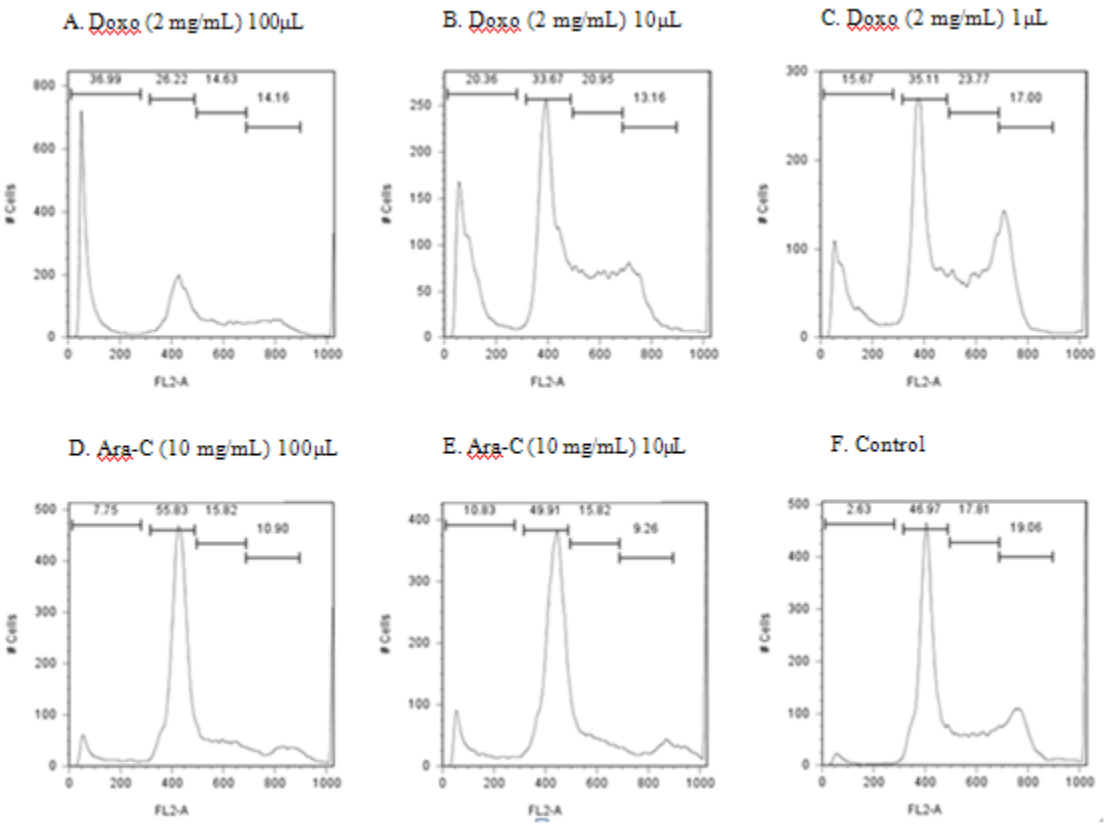


Figure-8: PI Staining of 3T3 Cells Treated With Graded Concentrations of Doxo and Ara-C. The majority of cells treated with Doxo are in an apoptotic state. The cells treated with Ara-C have the highest percentage of cells in G1 phase (cytostatic).

Table II: Percentages of 3T3 Cells in Each Cell Cycle Phase 24 Hours Post Drug Treatment.

	% of Total Cells			
Cells	**Apoptosis**	**G1**	**S**	**G2/M**
Control	2.19	45.82	17.09	17.14
100 µl Doxo	35.46	19.77	12.25	6.8
10 µl Doxo	18.6	31.03	17.36	10.93
1 µl Doxo	10.36	32.2	18.02	20.06
100 µl AraC	5.48	45.62	12.83	5.24
10 µl AraC	7.78	37.76	11.54	4.19

Figure-9 shows the FACS analysis of the PI staining of ME-1 cells treated with Doxo and Ara-C at various concentrations for 24 hours, and the values are quantitated in **Table III**. Cells were treated with Doxo at concentrations of 2 mg/mL, 0.4 mg/mL, 0.08 mg/mL and 0.016 mg/mL, and Ara-C at concentrations of 10 mg/mL, 2 mg/mL, 0.4 mg/mL, and 0.08 mg/mL. Panel A shows that the majority of ME-1 cells treated with 100 mg/mL of Doxo are in G2-M phase (right peak), and this pattern continues at the other Doxo amounts tested. This pattern is similar to untreated control cells, and to all concentrations of Ara-C tested, indicating neither drug had a strong effect on ME-1 cells.

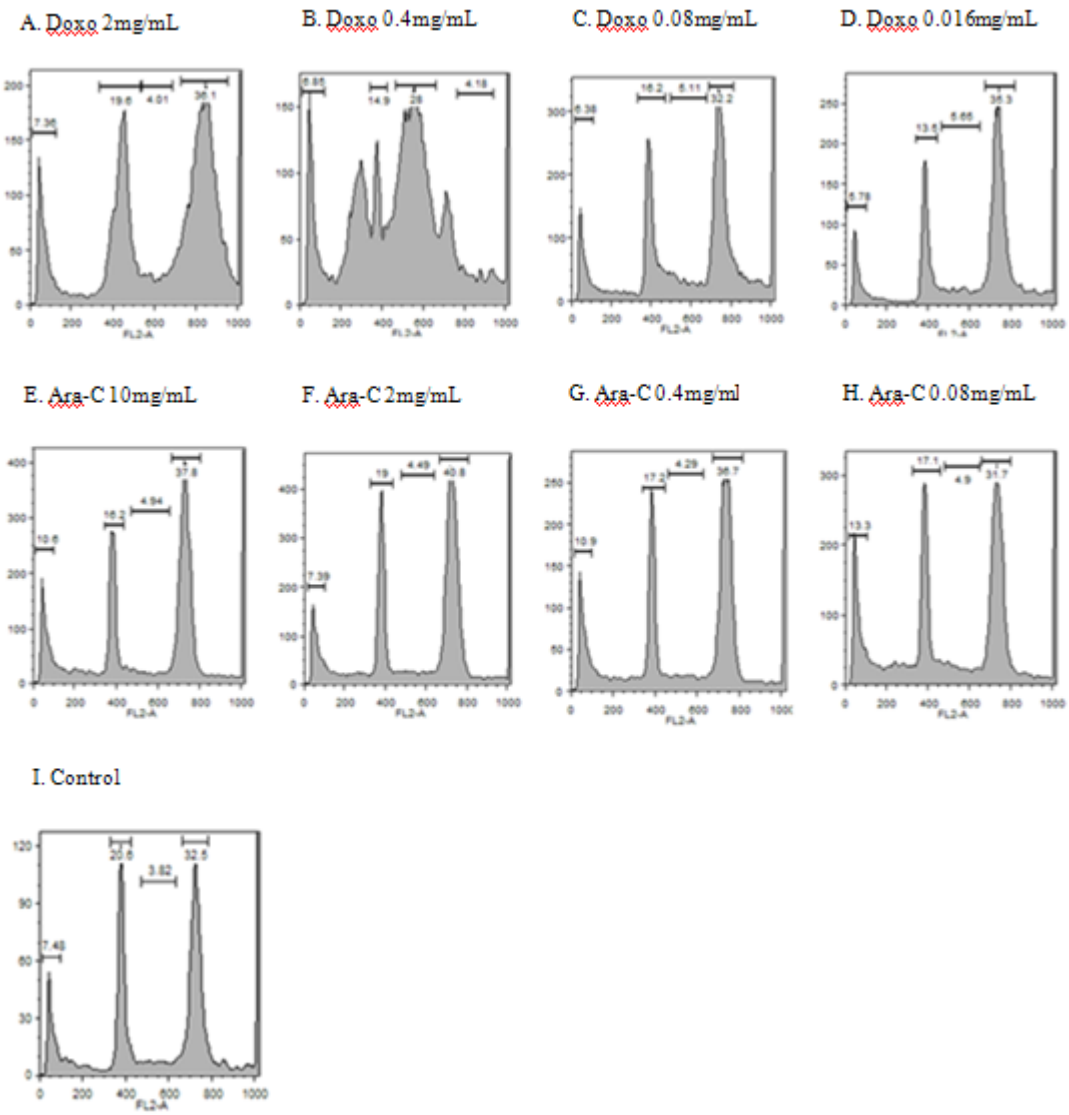


Figure-9: PI Staining of ME-1 Cells Treated With Graded Concentrations of Doxo and Ara-C. Neither drug had a strong observed effect on ME-1 cells at the concentrations tested.

Table III: Percentages of ME-1 Cells in Each Cell Cycle Phase 24 Hours Post Drug Treatment

	Total			
Cells	**Apoptosis**	**G1**	**S**	**G2/M**
ME-1 (Control)	7.48	20.6	3.82	32.5
1:1 Doxo	7.36	19.6	4.01	36.1
1:5 Doxo	6.85	14.9	4.18	28
1:25 Doxo	6.38	16.2	5.11	32.2
1:125 Doxo	5.78	13.5	5.65	35.3
1:1 Ara-C	10.6	16.2	4.94	37.8
1:5 Ara-C	7.39	19	4.49	40.8
1:25 Ara-C	10.9	17.2	4.29	36.7

Figure 10 shows the PI/FACS analysis on leukemic cells harvested ex vivo from an AML mouse model treated with either Doxo or Ara-C at various concentrations for 24 hours (Part A) and 48 hours (Part B). Cells were treated with Doxo at concentrations of 2 mg/mL, 0.4 mg/mL, 0.08 mg/mL and 0.016 mg/mL, and Ara-C at concentrations of 10 mg/mL, 2 mg/mL, 0.4 mg/mL, and 0.08 mg/mL. In each condition tested, the overwhelming majority of cells were in an apoptotic state, however even the control cells were in apoptosis, so it was not the drug which killed the cells, but rather another unknown factor.

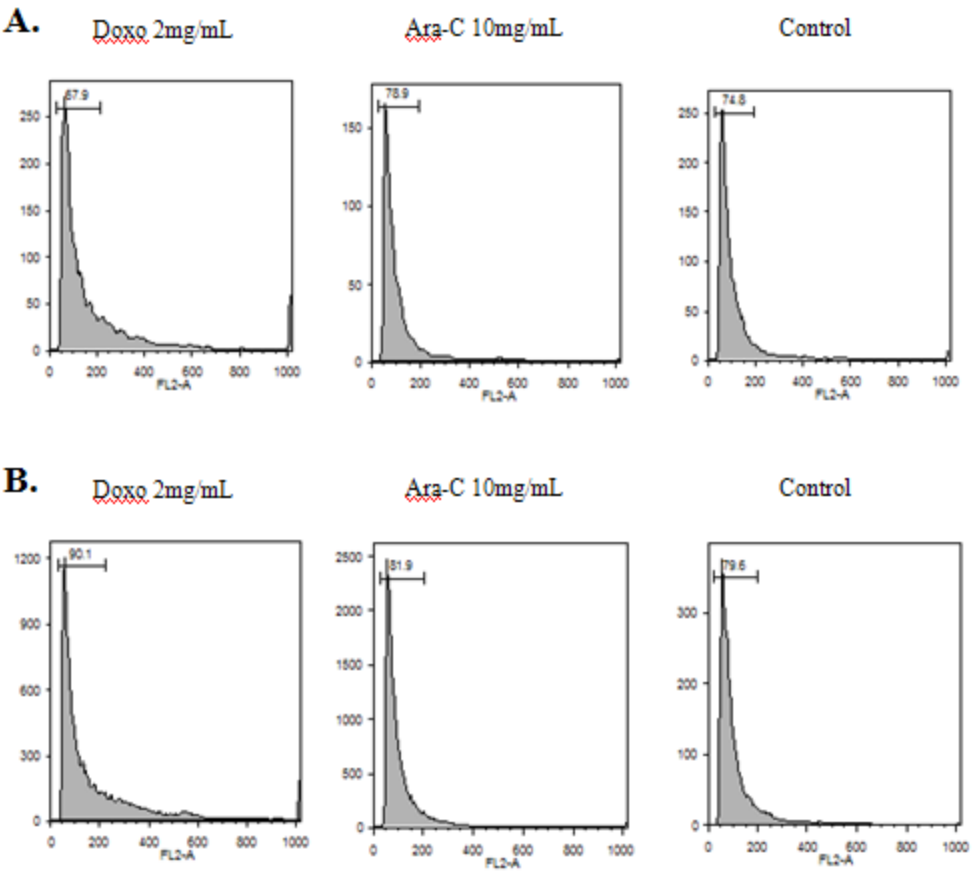


Figure 10: PI Staining of Leukemic Cells Taken *Ex Vivo* from an AML Mouse Model Treated With Graded Concentrations of Doxo or Ara-C. All cells were apopotic in this analysis, including untreated cells.

4.2 *In Vivo* Drug Testing: Toxicity of Doxo and Ara-C in Wild Type Mice

Previous studies have indicated that the two first-line chemotherapy drugs used to treat AML, Doxo and Ara-C, are extremely toxic because not only do they target preferentially expanding leukemic cells, but also normal proliferating cells. As a result, the optimum treatment regimes have not yet been established. Based on published murine studies, Doxo can be tolerated at 3 mg/kg every three days for three times (Lee et al, 2007). Additionally, Ara-C can be tolerated at 2 sessions of 100 mg/kg every day injected for nine days per session, with a seven

day rest period between sessions. Using this published schedule of treatment, a lethal concentration-25 (LC_{25}), the concentration of drug that kills 25% of the experimental population, is expected.

In this project, we began by validating the Lee et al (2006) study. The toxicity of each drug was established in wild type (WT) healthy mice. In all cases, toxicity was evaluated based on survival and daily inspection of the mice. Changes in motility, hair appearance, posture, and signs of anemia (indicated by pale paws) were also observed. When these physical signs were apparent, blood was analyzed for white blood cell (WBC) count, hematocrit (red blood cell (RBC) count), and platelets. When the toxicity of the drug caused these symptoms, mice were euthanized following procedure detailed in IACUC protocol A-1266 approved by the University of Massachusetts Medical School. However, since the mice survived the initial treatment and the expected LC_{25} results were not obtained (data not shown), different drug concentrations were tested to determine the highest tolerated dose (HTD) of each drug in WT mice, which would subsequently be used in the leukemia transplantation mouse model.

With respect to the HTD testing, an experiment was conducted to validate the previously published data that the HTD of Doxo in mice *in vivo* is 3mg/kg every three days injected three times. Two groups (n=4) were injected once every three days for a total of 9 days, at a dosage of 3 mg/kg or 2mg/kg of Doxo. If mice were able to survive the third dose, blood samples were analyzed for leukocytosis, hematocrit, and platelets (**Figure 11**). The data indicate that the mice given six dosages showed no physical signs of toxicity. Survival was not affected by the drug, as 100% of the treated mice remained alive (shown in **Table IV**). However, the results of the bleeding show that the average WBC count and platelet levels decreased slightly at the higher dose. These differences are negligible and there is no control for comparison. The standard

deviation for the 2 mg/kg was 0.53 for the WBC and 76.0 for the platelets. The standard deviation for the 3 mg/kg was 1.13 for the WBC and 46.39 for the platelets.

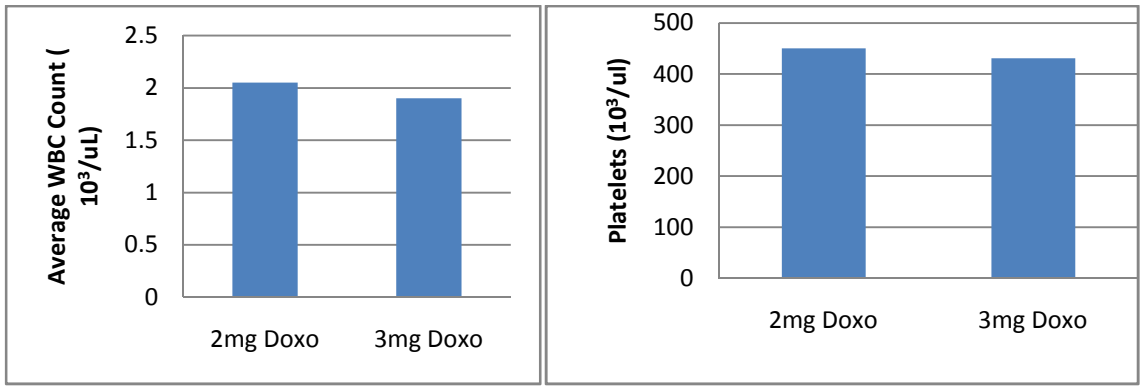


Figure 11: White Blood Cell Count (Left Panel) and Platelet Count (Right Panel) of WT Mice. The mice were treated with either 3 mg/kg or 2 mg/kg every 3 days for 9 days with Doxorubicin.

Table IV: Survival of WT Mice Treated with 3 mg/kg or 2 mg/kg Every Three Days for 9 Days with Doxorubicin.

	% of Living Mice	
Dose	**3 mg/kg/day Doxo**	**2 mg/kg/day Doxo**
1	100	100
2	100	100
3	100	100
4	100	100
5	100	100
6	100	100

The experiment described above was repeated, but with the addition of two groups of four mice. The first and second groups of mice were treated with 3 mg/kg and 2 mg/kg every three days for 27 days, along with the third group of mice treated with PBS, to serve as a control group. A fourth group was later added and treated with 4 mg/kg of Doxo every three days for 12 days (data not retrieved). The purpose of including the 4 mg/kg group was to test the tolerance

parameters of the mice. These mice were able to tolerate four injections of 4 mg/kg every three days. In this experiment, mice were able to survive past three dosages, up to nine total dosages (**Table V**). Although mice did have slightly affected motility, there were no other physical signs of toxicity, and survival was not affected. Blood analysis was performed after the third dose (**Figure 12**), and the mice were euthanized after the final fourth dosage. All mice, except for one mouse administered at the 3 mg/kg dosage, are below the normal range. There is no significant difference between the levels of WBCs for each group, including the PBS control. These cell and platelet counts may have been affected by the inaccuracy of the cell blood counter (CBC) machine. The standard deviation for the 2 mg/kg dosage was 0.39 for the WBC and 21.27 for the platelets. The standard deviation for the 3 mg/kg dosage was 0.61 for the WBC and 20.95 for the platelets.

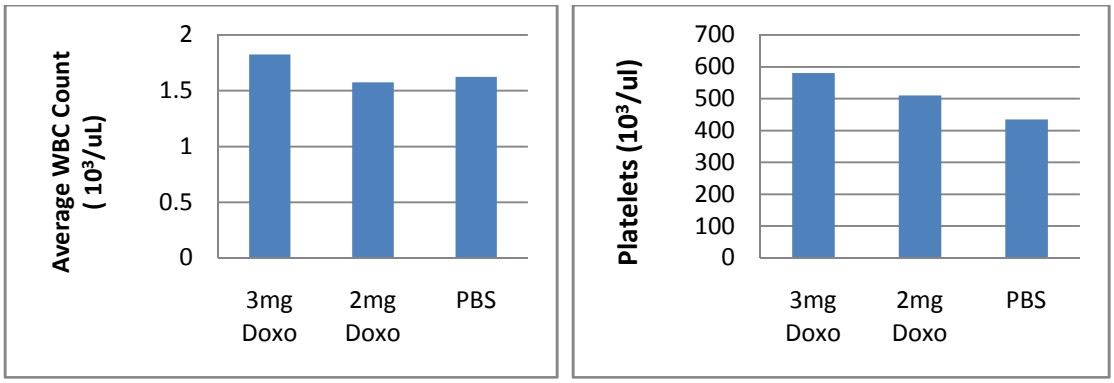


Figure 12: White Blood Cell Count and Platelet Count of WT Mice. Mice were treated with 3 mg/kg or 2 mg/kg every three days with Doxorubicin for 27 Days.

Table V: Survival of WT Mice Treated With 3mg/kg of Doxo, 2mg/kg of Doxo, or PBS, Every Third Day for 27 Days.

	% of Living Mice		
Dose	**3mg/kg/day Doxo**	**2mg/kg/day Doxo**	**PBS**
1	100	100	100
2	100	100	100
3	100	100	100
4	100	100	100
5	100	100	100
6	100	100	100
7	100	100	100
8	100	100	100
9	100	100	100

Since these dosage experiments were performed twice, and the toxicity of the drugs had no significant effect on the WT mice, we next tested a higher dose of 6 mg/kg every other day for ten total injections. After seven doses, the mice began to show signs of anemia (pale paws) and their coats became dull and scruffy. Also, their posture and motility decreased significantly. **Figure 13** shows the survival of the mice. All of the mice died by day 28.

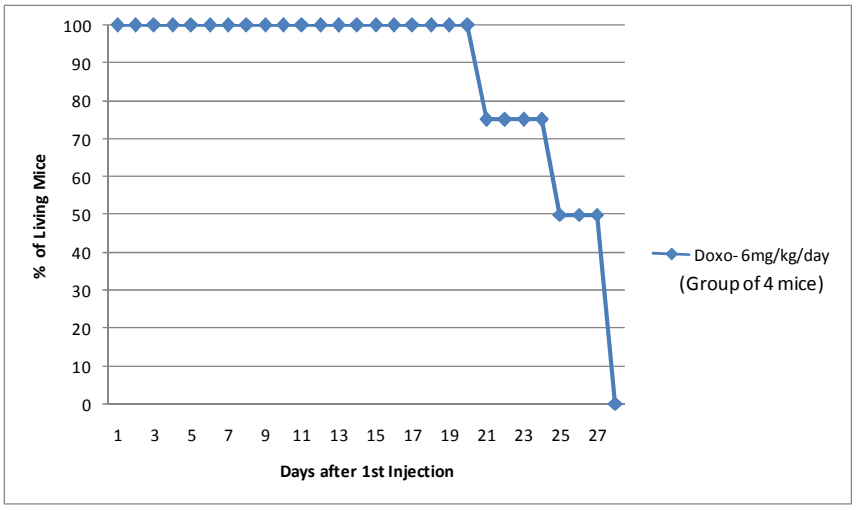


Figure 13: Kaplan-Meier Curve of WT Mice Treated With 6mg/kg Every Other Day with Doxo for 27 Days. All WT mice died after 27 days at this high Doxo dose.

With respect to Ara-C, according to published data mice can tolerate 2 sessions of 100 mg/kg every day for nine days per session, with a seven day rest between sessions (Largaespada, personal communication). In our initial Ara-C experiment (data not shown), mice were able to survive both 9-day sessions at a dosage of 100 mg/kg. Since our Doxo experiments allowed dosages much higher than previously published values, we also increased the dose of Ara-C to 200 mg/kg to a group of four mice (data not shown). The mice were able to tolerate the two 9-day sessions, so a third session was administered immediately following a fourteen day rest period between the second and third session. **Figure 14** shows the survival curve of this group of mice. The mice were administered a total of 27 dosages with an LC_{25} over a 48 day period.

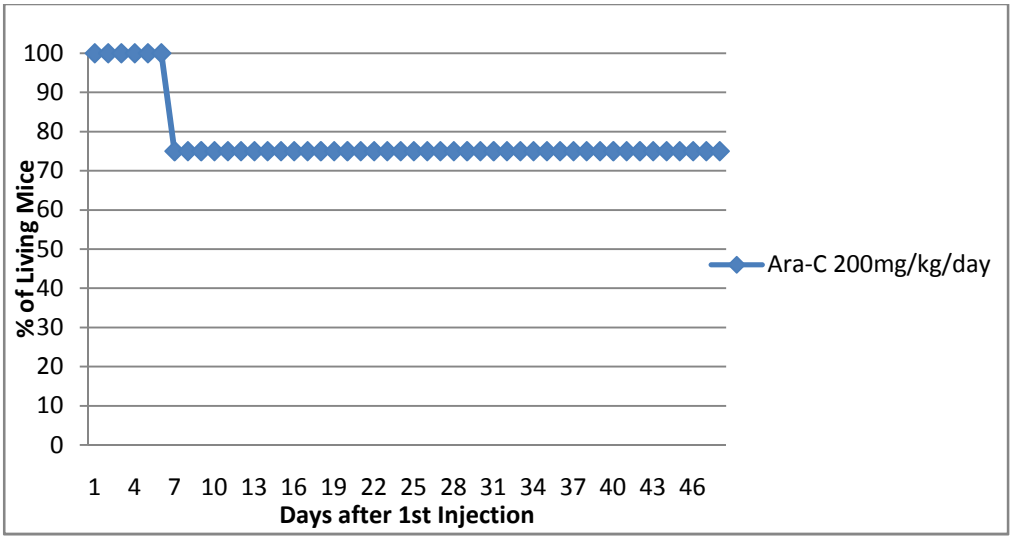


Figure 14: Kaplan-Meier Curve of WT Mice Treated with 200 mg/kg of Ara-C. The dose regime for Ara-C for this experiment was 200 mg/kg every day for nine days, then a 7 day rest period, then another 9-day injection session, then a 14-day rest period, then a third 9-day injection session (48 days total).

4.3 *In Vivo* Drug Testing: Efficacy and Toxicity of Doxo and Ara-C in the Leukemia Transplantation Model

The HTD of both Doxo and Ara-C established in section 4.2 was used to test the efficacy of each chemo drug in a mouse leukemia transplantation model. We tested the delay and recovery from leukemia development at the HTD of Doxo or Ara-C in 129 SvEv mice irradiated and transplanted with AML. Leukemia progression during and after chemo treatments was monitored by daily inspection of the phenotypic behavior of the mice, as well as analyzing blood samples through FACS, and cell morphology through microscopy of blood smears. The phenotypic behaviors that signify leukemia development include decreased mobility, distended abdomen (as a result of an enlarged spleen), and change in hair appearance (hair becomes discolored and dull). The mice were euthanized before the leukemia reached an advanced stage following the procedure detailed in the IACUC protocol for Dr. Castilla A-1266, approved by UMASSMED. Mice that died during the experiment were analyzed by necropsy for an enlarged spleen to confirm that leukemia caused their death.

Figure 15 shows the survival curves of mice treated with 300 mg/kg of Ara-C or 6 mg/kg/every other day of Doxo, exactly one week after irradiation and transplantation of leukemic cells. Based on the WT experiments with both Doxo and Ara-C, it was found that the mice were able to successfully tolerate the tested dosages. Therefore, the concentrations of both Doxo and Ara-C were increased for this experiment. Both groups achieved an $LC_{(0)}$ after 19 days of treatment. The 1 week leukemic Doxo mice were given a total of 6 doses, and survived for 19 days. The 1 week leukemic Ara-C mice were given a total of 7 doses and also survived 19 days.

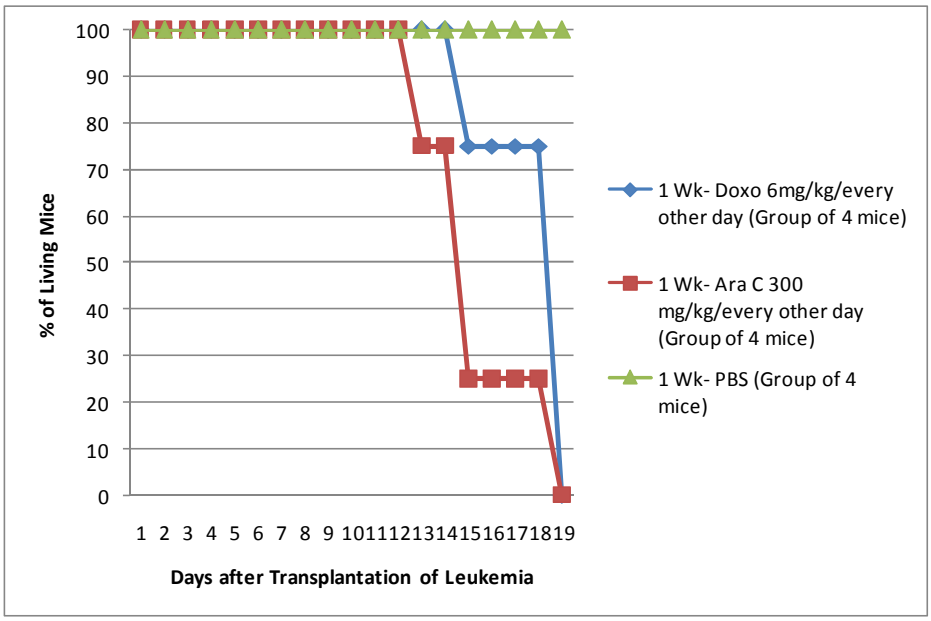


Figure 15: Kaplan Meier Curve of 1 Week Leukemic Mice Treated with Ara-C or Doxo. The dose regimes for these mice were 300 mg/kg Ara-C or 6 mg/kg Doxo, every other day, for 19 days.

Figure 16 shows three groups of leukemic mice administered treatment with PBS, Ara-C or Doxo as in Figure 15, except administration began exactly 4 weeks post transplantation. The group treated with Ara-C was given 9 doses every day, completing 1 session of treatment, and surviving a total of 38 days with an $LC_{(0)}$. The mice treated with Doxo were given 6 doses and survived a total of 39 days with an $LC_{(0)}$.

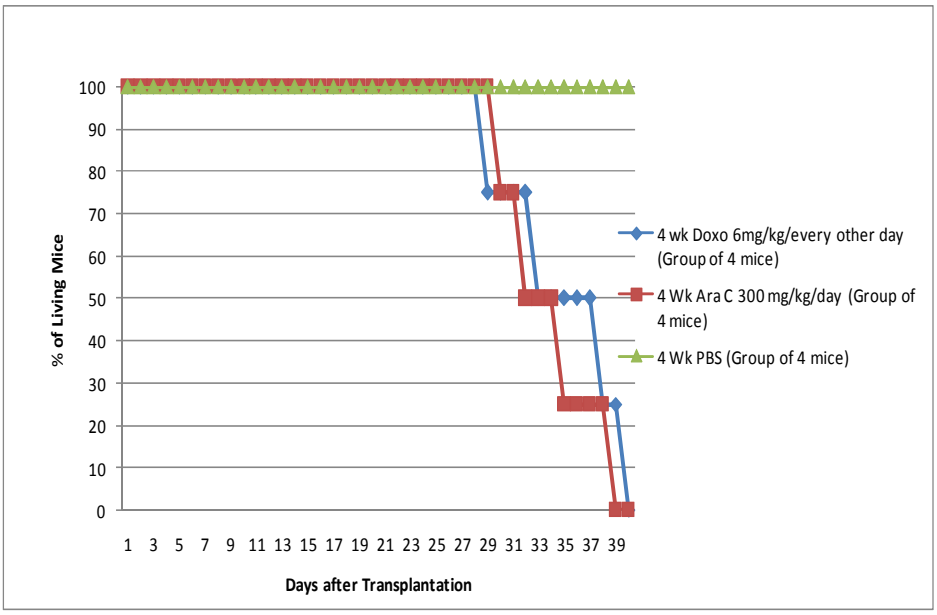


Figure 16: Kaplan-Meier Curve of Leukemic Mice Treated 4 Weeks After Transplantation with PBS, Doxo, or Ara-C. The drug dosages were as described in the previous figure.

Figure 17 shows the FACS analysis of peripheral blood and bone marrow samples stained for cKit which indicates the presence of leukemia, from a mouse transplanted with AML and treated with Doxo 4 weeks post transplantation. The pink box on the right shows the percentage of cells that are positive for cKit. As shown in Figure 17, less than 1% of the cells were positive for leukemia. Therefore, the mice did not die of leukemia.

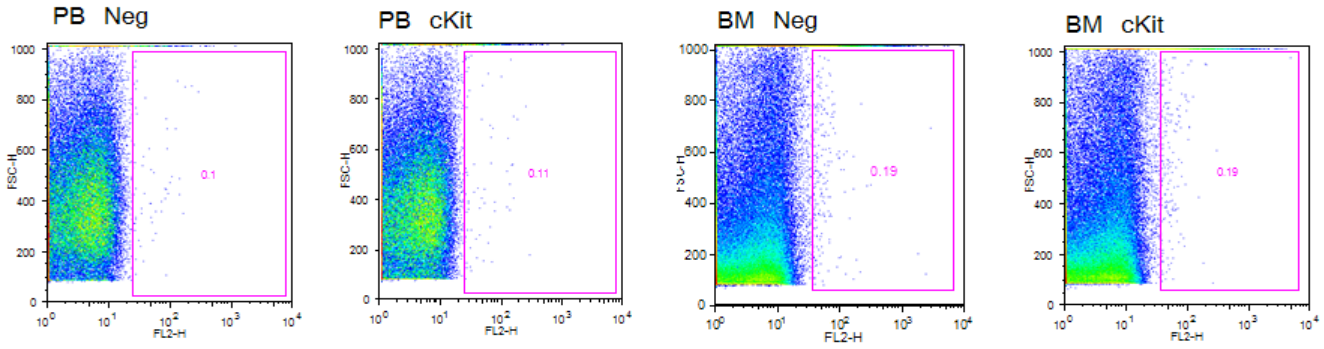


Figure 17: FACS Analysis of Peripheral Blood and Bone Marrow Cells in a 4 Week Post-Transplantation Leukemic Mouse Treated with Doxo. The drug regime was as described in the previous figure.

Figure 18 shows the FACS analysis of peripheral blood samples stained for cKit from another mouse transplanted with AML and treated with Doxo 4 weeks post transplantation. The pink box on the right shows the percentage of cells that are positive for cKit, which indicates the presence of leukemia.

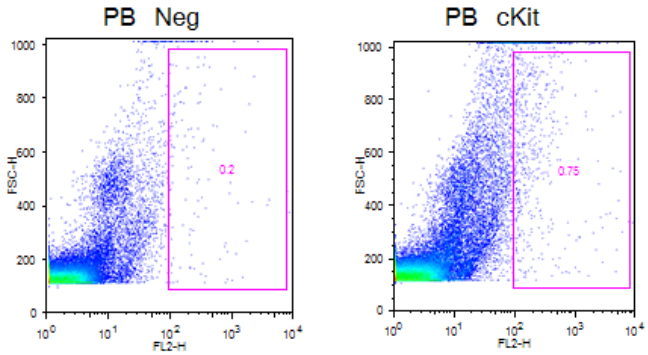


Figure 18: FACS Analysis of 4 Week Leukemic Mouse Treated with Doxo.

Figure 19 shows FACS analysis of peripheral blood samples stained for cKit from a mouse transplanted with AML and treated with PBS 4 weeks post transplantation. The pink box on the right shows the percentage of cells that are positive for cKit, which indicates the presence

of leukemia. In the mouse treated with PBS, note the strong appearance of c-Kit+ leukemic cells in the peripheral blood (4.02%) compared to 0.75% for a mouse treated with Doxo.

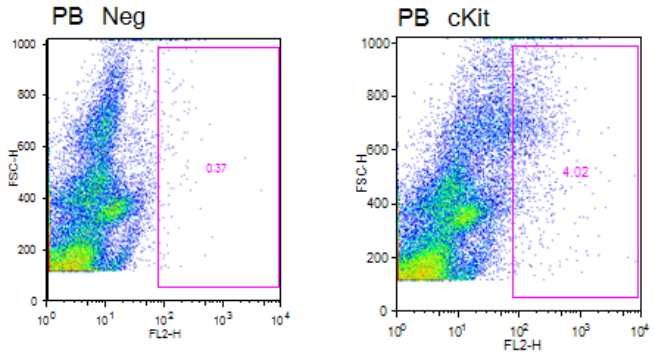
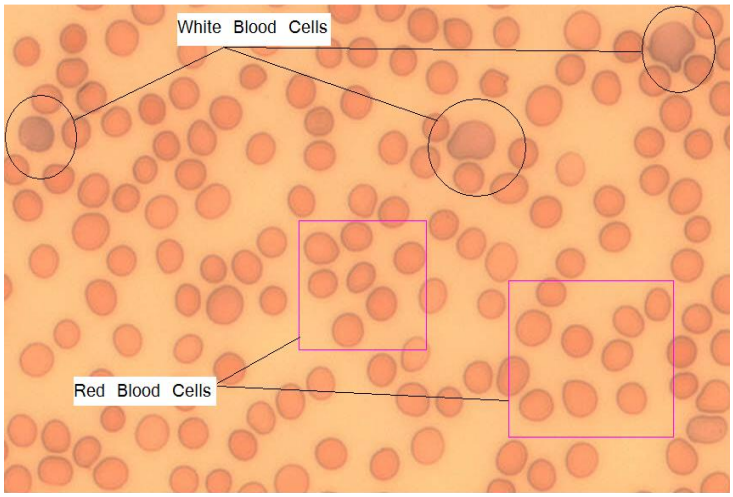


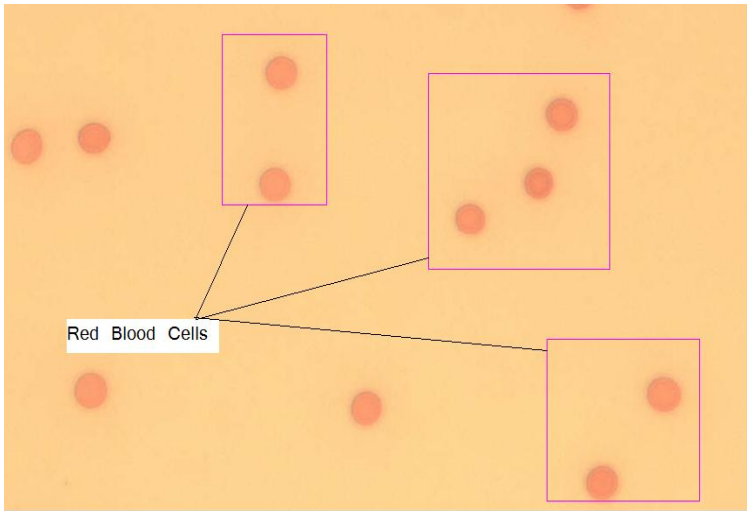
Figure 19: FACS Analysis of 4 Week Leukemic Mouse Treated with PBS.

Blood samples were collected from mice to analyze the cell morphology to determine if they were dying from leukemia, or from drug toxicity (**Figure 20**). White blood cells were only observed in the mice receiving PBS treatment (Panel A), not in mice receiving Doxo (Panel B) or Ara-C (Panel C). Thus it was determined that the drug-treated mice were dying from cytopenia, specifically low white blood cell counts, or leukopenia.

A.



B.



C.

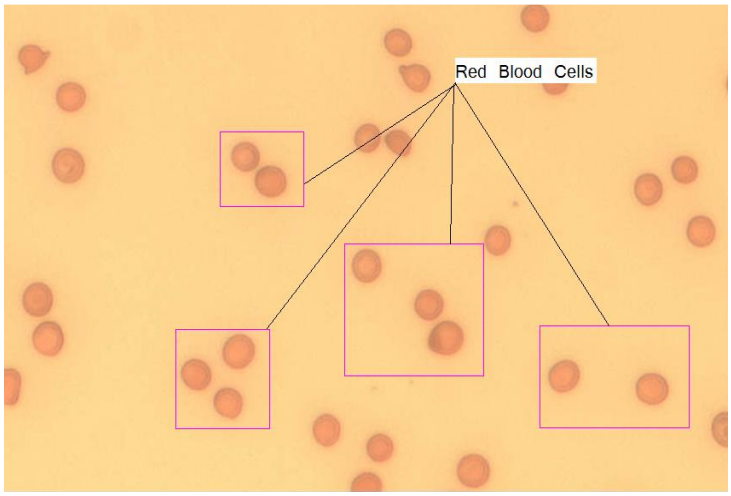


Figure 20: Microscopy of Blood Smears of Leukemic Mice Treated 4 Weeks Post Transplantation (40x). A. Blood smear of leukemic mouse treated with PBS 4 weeks post transplantation. Examples of white blood cells are in circles, and examples of red blood cells are in squares. There is a normal distribution of white blood cells among red blood cells. **B.** Blood smear of leukemic mouse treated with Doxo 4 weeks post transplantation. There are no white blood cells present on the slide, indicating an extremely low presence of WBCs in the mouse's bloodstream. **C.** Blood smear of leukemic mouse treated with Ara-C 4 weeks post transplantation. There are no white blood cells present on the slide, indicating an extremely low presence of WBCs in the mouse's bloodstream.

In order to compare the timing of the Doxo treatment relative to the length of transplantation of the leukemic cells, two groups containing four mice each were treated with the previously established HTD of Doxo (6 mg/kg/every other day), one group was treated 1 week after irradiation and transplantation with $5x10^5$ leukemic cells, while the second group was treated 4 weeks after irradiation and transplantation with $5x10^5$ leukemic cells (**Figure 21**). The first group had and $LC_{(100)}$ after 19 days of treatment, and the second group had an $LC_{(100)}$ after 40 days of treatment. A third group of four mice was treated with PBS, and mice survived past day 40, with 100% survival. Thus, treatment with Doxo at 6 mg/kg killed the mice faster if the mice were only 1 week post-leukemic cell transplantation.

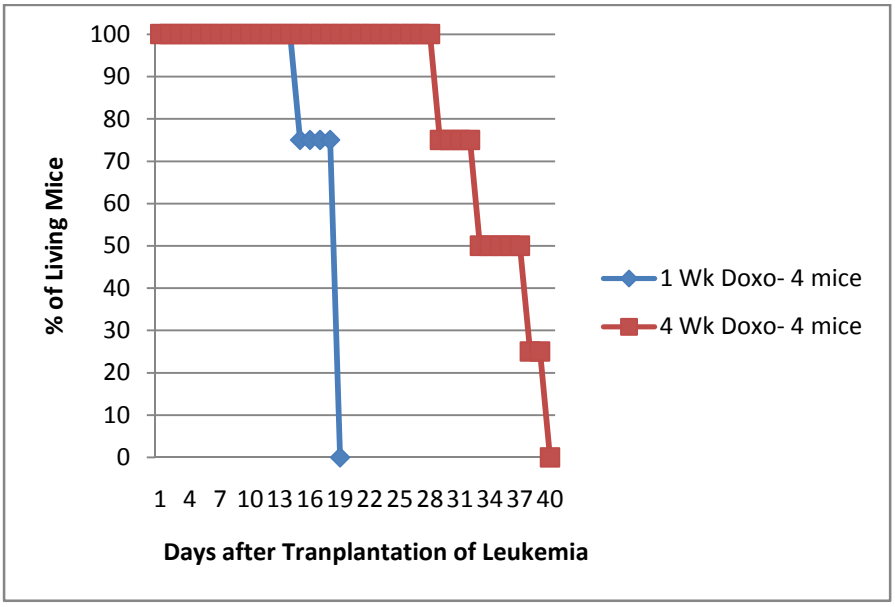


Figure 21: Kaplan-Meier Curve of 1 Week vs. 4 Week Leukemic Mice Treated with Doxo.

In order to compare the Ara-C treatment with time of leukemic cell transplantation, two groups with four mice each were treated with the previously established HTD of Ara-C (300 mg/kg/every day), one group was treated exactly 1 week after irradiation and transplantation with $5x10^5$ leukemic cells, while the second group was treated 4 weeks after irradiation and transplantation with $5x10^5$ leukemic cells (**Figure 22**). The first group had an $LC_{(100)}$ after 19 days of treatment, and the second group had an $LC_{(100)}$ after 39 days of treatment. The third group of four mice treated with PBS all survived past day 40, with 100% survival.

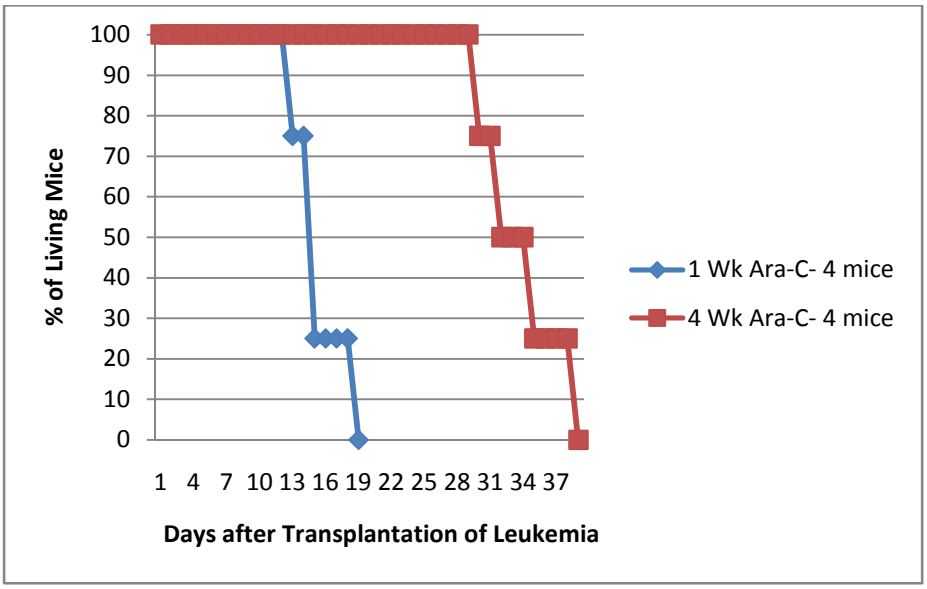


Figure 22: Kaplan-Meier Curve of 1 Week Versus 4 Week Leukemic Mice Treated with Ara-C.

The peripheral blood of the mice in the Ara-C experiment of Figure 22 was analyzed by FACS for c-Kit (**Figure 23**). The pink box on the right shows the percentage of cells positive for cKit, which indicates the presence of leukemia. Since less than 1% of the cells contain leukemia, the mice died from other causes.

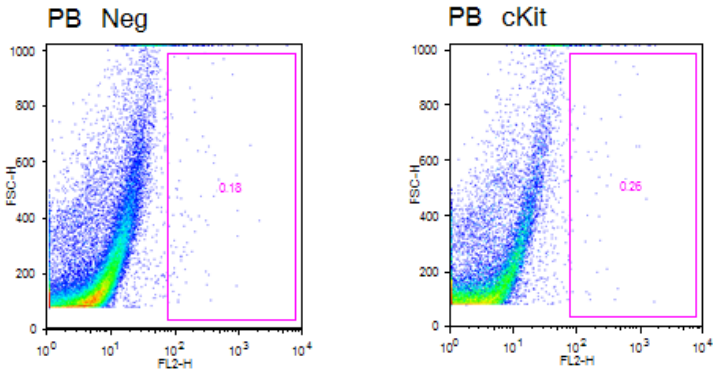


Figure 23: FACS Analysis of 4 Week Leukemic Mouse Treated with Ara-C.

Because all leukemic mice in each chemo group died in the previous experiments, new groups of mice to be injected with lower doses of chemo drugs were established. As there was only time to study one drug at this point during the project, Ara-C was chosen. The dosage concentration of Ara-C was lowered from 300 to 200 mg/kg, however the number and frequency of doses was kept the same. Also, another group was added to test the possible toxic combination of irradiation and treatment with Ara-C on the mice. The three groups of mice tested included a group of 6 mice irradiated and transplanted with $5x10^5$ leukemic cells, and administered 200 mg/kg of Ara-C, a second group of 5 mice that was irradiated only, not transplanted with leukemia, and administered the same dose of Ara-C, and a third group that was irradiated and transplanted with $5x10^5$ leukemic cells and treated daily with PBS.

Figure 24 shows the survival of these mice. The first group, which consisted of 6 mice that were irradiated and transplanted with leukemia, was given 9 doses, and survived for 16 days with an $LC_{(100)}$. The second group, which consisted of 5 mice that were irradiated only, were given 8 doses and survived for 15 days with an $LC_{(100)}$. The group of irradiated and transplanted mice treated with PBS survived for 16 days with an $LC_{(0)}$. The irradiated non-transplanted Ara-C mice died as quickly as the irradiated transplanted Ara-C mice, while the irradiated transplanted non drug treated mice survived, indicating the radiation treatment coupled with the toxic drug may severely weaken the mice.

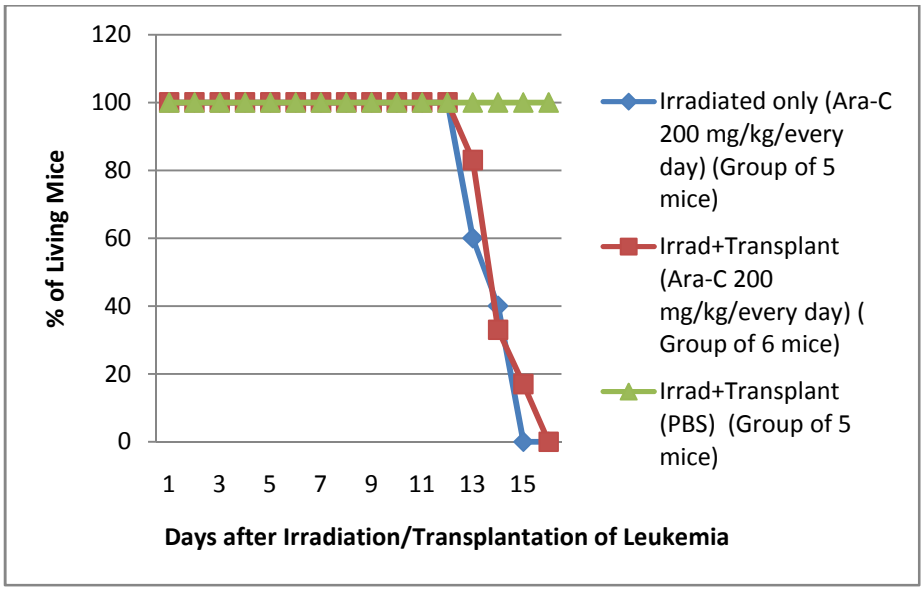


Figure 24: Kaplan-Meier Curve of Irradiated/Transplanted Mice Versus Irradiated-Only Mice Treated with Ara-C.

A final experiment was performed to determine if the mice would survive longer simply by administering the dose of 200 mg/kg of Ara-C every other day instead of every day. Group-1 contained 8 mice, irradiated and transplanted with $5x10^5$ leukemic cells, and treated with 200 mg/kg/every other day of Ara-C. Group-2 contained 8 mice, irradiated and transplanted with $5x10^5$ leukemic cells, treated with 150 mg/kg/every other day of Ara-C.

Group 3 contained 9 mice, irradiated only, and treated with 200 mg/kg/every other day of Ara-C.

Group 4 contained 7 mice, irradiated and transplanted with $5x10^5$ leukemic cells, treated with PBS every other day. **Figure 25** shows the survival of the four groups. Groups 1 and 3 survived the first session of treatment with an $LC_{(25)}$, which is a significant improvement from the $LC_{(100)}$ of the previous experiment.

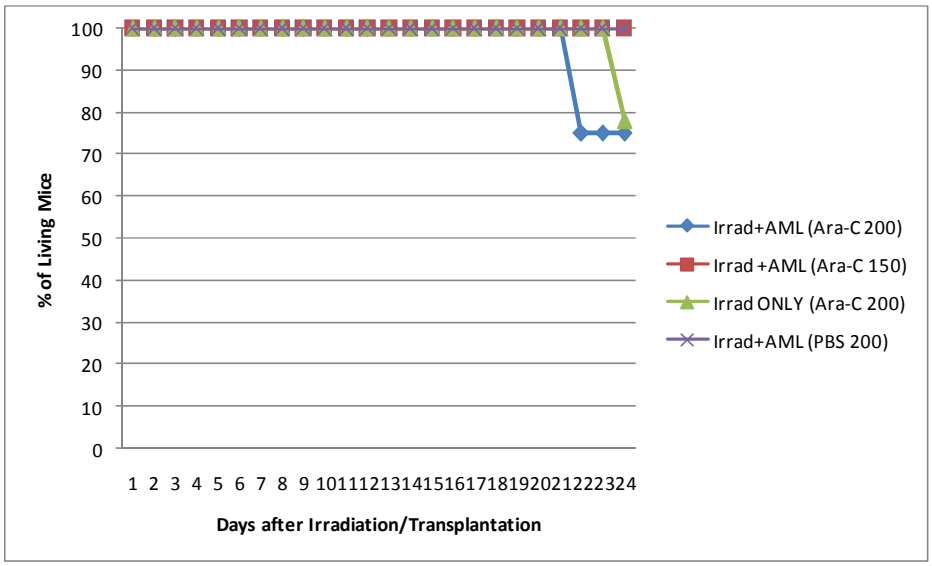


Figure 25: Kaplan-Meier Curve of Leukemic Mice and Irradiated Control Mice Treated with 200 mg/kg of Ara-C.

Table VI shows the organ weight in grams of mice sacrificed or found dead in this entire project. Organ weight data was collected from the 1 week post transplantation group treated with Doxo, the 1 week post transplantation group treated with Ara-C, the 4 week post transplantation group treated with Doxo, the 4 week post transplantation group treated with Ara-C, the 4 week post transplantation group treated with PBS, the first set of mice that were irradiated only and treated with Ara-C, the first set of mice that were irradiated, transplanted with AML, and treated with Ara-C, the second set of mice that were irradiated only and treated with Ara-C, and the second set of mice that were irradiated, transplanted with AML, and treated with Ara-C. Through necropsy, the spleen, stomach, and liver were extracted and weighed, to identify the cause of death of the mouse. An enlarged spleen signifies death by leukemia, whereas an enlarged stomach or liver signifies death by drug toxicity. The necropsy of the mice treated 4 weeks post transplantation indicated the majority of mice treated with Doxo or Ara-C

had a spleen weight of approximately 0.1 gm, while mice treated with PBS had a spleen weight of approximately 0.5 gm. The average spleen weight of a wild type mouse is 0.07-0.1g (Yan et al, 2002). Since an enlarged spleen often characterizes the presence of leukemia, this suggests that mice treated with the chemo drugs did not die from AML.

Table VI: Necropsy Data-Organ Weights.

Type of Mouse		*Weight (grams)*		
		Spleen	Liver	Stomach
1 week Doxo		-	-	1.43
1 week Ara-C		-	-	3.11
1 week Ara-C		-	-	2.62
4 week Ara-C		0.105	-	-
4 week Doxo		0.118	-	-
4 week Doxo		0.158	-	-
4 week Ara-C		0.813	1.57	-
4 week Ara-C		0.23	1.08	-
4 week Doxo		0.063	-	-
4 week PBS		0.48	-	-
1. Irr only+Ara-C		0.006	1.07	3.55
1. Irr only+Ara-C		0.019	0.82	2.9
1. Irr only+Ara-C		0.06	0.67	2.71
1. Irr only+Ara-C		0.08	0.86	2.11
1. Irr only+Ara-C		0.05	1.09	2.83
1. Irr+AML+Ara-C		0.01	0.712	2.76
1. Irr+AML+Ara-C		0.028	0.83	1.85
1. Irr+AML+Ara-C		0.019	0.58	0.82
1. Irr+AML+Ara-C		0.01	1	1.68
1. Irr+AML+Ara-C		0.38	0.81	1.88
1. Irr+AML+Ara-C		0.1	0.98	0.6
2. Irr+AML+Ara-C		0.045	0.687	0.431
2. Irr+AML+Ara-C		0.063	0.429	0.532
2. Irr only+Ara-C		0.05	0.71	0.61
2. Irr only+Ara-C		0.008	0.78	0.55

5.0 DISCUSSION

The primary goal of this project was to design a Doxo or Ara-C treatment regime to decrease the toxic effect of these key chemotherapy drugs in the leukemia transplantation mouse model while increasing drug efficacy. This goal was accomplished by establishing an appropriate drug concentration, dose and schedule. In order to accomplish the primary goal of the project, experiments were conducted in three stages. The first stage involved *in vitro* drug testing using 3T3, ME-1, and mouse leukemic cells. The second stage included *in vivo* drug testing with WT mice. The third stage of the project included *in vivo* drug testing in the leukemia transplantation model.

5.1 *In Vitro* Drug Testing

During the *in vitro* experiments, 3T3 cells were used as a marker for comparison with ME-1 cells. It was expected that the 3T3 cells would be affected by the chemotherapy drugs, as 3T3 cells have a rapidly proliferating cell cycle that can double in 24 hours. Doxo had a cytotoxic effect on the 3T3 cell line, as the majority of these cells were in the apoptotic state of the cell cycle. Ara-C had a cytostatic effect on this cell line, as the majority of these cells were in the G1 phase of the cell cycle. There was little difference between the cells treated with a volume of 10 μL of Ara-C in G1 phase and control cells in G1 phase. Therefore in future experiments, lesser volumes of Ara-C can be used to achieve the same result.

It was originally expected that the ME-1 cells, which carry the inv(16)mutation that produces AML, would be affected by the various concentrations of Doxo and Ara-C after the 24 hour incubation period. However, the ME-1 cells were unaffected by 24 hour incubation with the

chemotherapy drugs because the cell cycle is longer than 24 hours. While chemotherapy drugs are cytotoxic and, since ME-1 and some other AML cells have "frequently low DNA synthesis rates", it would take an extended period of time for the chemotherapy drugs to have an effect on their cell cycles (Butturini, 1990). This experiment was repeated to determine how these drugs affect the cell cycle at various incubation time points. PI analysis was performed at 24 hours, 48 hours, 72 hours, and 120 hours after initial incubation with Doxo and Ara-C. However, we were unable to obtain results from these experiments with extended time points because there were not enough cells to be analyzed through FACS analysis due to difficulty culturing ME-1 cells. In the future, it may be beneficial to repeat these experiments with a stock of ME-1 cells that will survive throughout the various incubation time points.

The same experiment was repeated with leukemic cells harvested from a mouse with AML and we were not able to obtain results due to cell death in culture. This may have been caused by the decrease in viability over time of the cryopreserved cells. The procedures for cryopreserving and culturing these leukemic cells need to be fine-tuned in order to ensure cell growth and survival throughout the duration of the experiment.

5.2 *In Vivo D*rug Testing in WT Mice

In order to gain a better understanding of the toxicity and efficacy of Doxo and Ara-C as treatment options for AML, toxicity experiments were first conducted on WT mice. The dosage concentrations and schedule found to work most efficiently with little toxic effect on the mice differed from previously published data. It was previously determined by *Lee et. al* that mice are able to tolerate 3 mg/kg every three days for three dosages of Doxo. However, we found that WT mice can tolerate up to 6 mg/kg every other day for ten dosages of Doxo. The strain of mice used

in the two experiments varied, which led us to conclude that the WT 129 SvEv mouse strain used in this project is less sensitive to the toxicity of these drugs and can tolerate a much higher dosage of Doxo for an extended period of time than the FVB/N mice used in the *Lee et. al* study.

Similarly, during the Ara-C toxicity experiments in WT mice, we found that the 129 SvEv mice could tolerate higher concentrations over a longer period of time than the strains of mice used in previously published studies. In a study by Largaespada, mice were able to tolerate two sessions of 9 injections with 100 mg/kg of Ara-C, with a one week rest period between the two sessions. The mice from the published data had an $LC_{(25)}$, and while the 129 SvEv mice we analyzed also had an $LC_{(25)}$, the dosage concentration and schedule exceeded the study from the published data. The 129 SvEv mice used in our experiments were given three sessions of 9 injections with 200 mg/kg of Ara-C, with a one week rest period between the first and second sessions, and a fourteen day rest between the second and third sessions. Again, this proved that the WT 129 SvEv strain of mice have a higher tolerance for Doxo and Ara-C treatments. It appears that we cannot transfer data about drug tolerance from one strain to another. In addition, the dosages and concentrations of treatment from previously published studies were performed on mice that were irradiated and transplanted with leukemic cells. In our toxicity experiments, the effect of irradiation on the mice was not taken into account, which may be an explanation as to why the WT 129 SvEv mice were able to tolerate such high doses of the chemotherapy drugs for an extended length of time.

5.3 *In Vivo D*rug Testing in the Leukemia Transplantation Model

The concentrations and dosage schedules determined in wild type mice were used as a starting point to establish treatment with chemotherapy drugs in the mice transplanted with

AML. We wanted to observe the efficacy of each drug at preventing the progression of leukemia, and in the treatment of leukemia after it had developed to a more advanced stage. Therefore, we began injections at two different time points, 1 week post transplantation and 4 weeks post transplantation. The mice that were treated 1 week post transplantation died only 19 days after the initial transplantation, while the mice treated after 4 weeks post transplantation died after 39 days. A possible reason why the 1 week post transplantation mice died so quickly may be a result of the irradiation causing the immune systems of the mice to become weakened and fragile. The 4 week post transplantation mice had an additional three weeks to recover from irradiation and strengthen their immune systems to be able to better tolerate the toxicity of the chemo drugs. It was determined that from a survival perspective, it would be more efficient to administer chemotherapy treatment four weeks after irradiation.

The necropsy of the mice treated 4 weeks post transplantation indicated the majority of mice treated with Doxo and Ara-C had a spleen weight of approximately 0.1g, while mice treated with PBS had a spleen weight of approximately 0.5g. The average spleen weight of a WT mouse is 0.07-0.1g (Yan et al, 2002). Since an enlarged spleen often characterizes the presence of leukemia, this suggests that mice treated with the chemo drugs did not die from AML. It is apparent from the enlarged spleen weight, as well as the presence of cells positive for cKit, the marker for leukemic cells, that the mice treated with PBS died of leukemia.

From analysis of cell morphology, it was determined that mice treated with Doxo and Ara-C 4 weeks post transplantation had extremely low white blood cell counts. Since leukemia did not appear to be the cause of death for these mice, this led to the conclusion that the mice died of cytopenia, most likely caused by the toxicity from the treatments with Doxo and Ara-C.

In order to gain better insight into the cause of death of the mice, a second set of experiments was performed, however, only Ara-C was used as a treatment due to the time restraints of the project. During these experiments, three groups of mice were treated. These groups included the first group of mice that was irradiated, transplanted with AML and treated with 200 mg/kg of Ara-C, a second group that was irradiated-only and treated with 200 mg/kg of Ara-C, and a third group that was irradiated, transplanted with AML and treated with PBS. The first two groups began dying after the 6^{th} injection, and all mice in this group were dead by the 9^{th} injection. The second group, that was irradiated but was not injected with AML cells, served as a control to determine if the combination of irradiation and drug toxicity was leading to the premature death of the mice. Since this second group died at the same time and rate as the first group, this proved that it was the combination of the toxicity of the drug and irradiation which killed them as opposed to the combination of the toxicity of the drug and the leukemia that was originally thought to be killing the mice.

As a result of the mice dying after the 9^{th} injection, similar experiments were performed treating the mice every other day with 200 mg/kg instead of treating the mice daily with Ara-C. We also added a second group of mice treated with only 150 mg/kg every other day. This helped to determine if the daily dosages of 200 mg/kg were too toxic on the internal systems of the mouse's body. We determined that the mice treated with 200 mg/kg every other day survived with a $LC_{(25)}$ up to the 9^{th} injection at day 24, compared to the $LC_{(100)}$ at the 9^{th} injection at day 16 for the mice treated every day. The 150 mg/kg had an $LC_{(0)}$ up to the 9^{th} injection by day 24. Therefore, the treatment of 200 mg/kg of Ara-C every other day was less toxic to the mice, and this combination of irradiation and treatment increased mouse survival by 75% after 5 injections.

The results obtained in these experiments support the conclusion that a balance between drug toxicity and AML prevention must be found. Although these drugs killed the leukemic cells, they also killed normal, proliferating cells resulting in mouse death. While the wild type mice were able to tolerate high dosages of Doxo and Ara-C, the irradiation enhanced the toxic effects of each drug in the leukemic model. However, when the dosages of each drug were lowered and the mice were given time to recover from the irradiation, the mice survived longer without showing signs of leukemia. Our research shows that two important factors in decreasing the toxicity and increasing the efficacy of AML treatment are the amount of recovery time post irradiation and the concentration of drug dose.

In order to improve treatment for patients with AML, a balance between drug toxicity and efficacy must be determined. This established treatment can be combined with other compounds, which could potentially inhibit activity of the oncoprotein CBFβ-SMMHC. The Doxo and Ara-C compounds tested here were previously found by a high through put screen and have the potential to specifically target AML cells. With a combined treatment the chemo drugs might eliminate the existing leukemic cells and prevent the formation of new leukemic cells expressing the oncoprotein CBFβ-SMMHC. We hope that these findings contribute to potential new therapies that will improve the long term remission rates of patients suffering from AML.

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